CXLVI. THE DETERMINATION OF PYRUVIC ACID.

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(*Received May 25th, 1928.*)

The methods employed for the estimation of pyruvic acid are largely based upon the reaction of its carbonyl group with phenylhydrazine. Its estimation in complex biological fluids where there are other compounds which react likewise is therefore difficult and, particularly when pyruvic acid exists in small quantities amidst these compounds, the estimation is unreliable even when controls are run. This difficulty was greatly felt in some of our investigations, and it was thought worth while to study some of the existing methods which could be satisfactorily employed as such or modified for our purpose.

The production of an intense blue colour by sodium nitroprusside and ammonia, especially in the presence of a little acetic acid, has been claimed to be specific for pyruvic acid and unaffected by acetaldehyde.

The precipitation of pyruvic acid as hydrazone by excess of phenylhydrazine followed by the determination of the excess phenylhydrazine has been the subject of considerable study [Smedley-MacLean, 1913; Simon and Piaux, 1924].

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid and the subsequent estimation of lactic acid according to the method of Fürth and Charnass has been adopted by Lieben [1923].

The oxidation of pyruvic to acetic acid by a slight excess of hydrogen peroxide has been shown to be quantitative by Holleman [1904], and potassium dichromate has been employed similarly and the excess dichromate estimated.

As it is not the purpose of this paper to give the results of the comparative study of these methods, it will be sufficient to say that none of them as such was found suitable in our experiments, and all required some modifications before they could be employed.

The necessary conditions for a method which would be suitable to our requirements were that it should be applicable to small quantities of the acid, usually between 0·5 and 15 mg., and in solutions of very low concentration, *i.e.* 0·1 % to 0·05 %.

The method finally developed is a modification of Lieben's technique, and is based upon the following reactions:

$$CH_3.CO.COOH + 2H = CH_3.CHOH.COOH \quad \text{.....(i).}$$

$$CH_3.CHOH.COOH + O = CH_3.CHO + CO_2 + H_2O \quad \text{.....(ii).}$$

The reduction of pyruvic to lactic acid by zinc and hydrochloric acid has been shown to be quantitative by Lieben. The lactic acid was estimated according to Fürth and Charnass. A micro-adaptation of Fürth and Charnass's method, with small quantities of pyruvic acid reduced by zinc and hydrochloric acid, gave very irregular results, due probably to very slight but irregular decomposition of pyruvic acid when boiled for a long time with 10 % HCl. With higher concentrations of pyruvic acid the results are of the same degree of accuracy as those of Lieben, and, with an empirical factor for lactic acid, its equivalent of pyruvic acid can be accurately calculated. Some of the results obtained according to the micro-adaptation of Fürth and Charnass's method of oxidation, and for larger amounts of acid according to the original method, are given in Tables I and II respectively. In all these cases, however, the titration of bound aldehyde was made according to Clausen [1922].

Table I.

Different amounts of pyruvic acid were reduced by boiling with 50 cc. of 10 % HCl and 0·5 to 0·75 g. of zinc for 2¾ hours.

Pyruvic acid taken (mg.)	0·68	1·36	1·36	2·04	2·04	3·40	3·40	2·72
Found (%)	79·4	85·3	81·6	85·3	82·9	86·5	82·9	83·1
Pyruvic acid taken (mg.)	2·72	6·80	6·80	10·20	10·20	13·60	13·60	
Found (%)	84·6	85·5	82·0	86·2	88·8	89·2	87·1	

According to the same method Clausen finds with lactic acid a yield on an average of approximately 92 %, about the same as that found for larger quantities. For comparison some results of the estimation of larger quantities of pyruvic acid according to the original method of Fürth and Charnass are given in Table II.

Table II.

Different amounts of pyruvic acid were reduced by boiling with 100 cc. of 10 % HCl and 2 g. of zinc for 2¾ hours.

Pyruvic acid taken (mg.)	12·14	12·14	24·28	24·28	36·42	36·42	48·56
Found (%)	86·9	85·8	86·7	90·2	88·3	87·9	88·5
Pyruvic acid taken (mg.)	48·56	60·7	60·7	121·4	121·4	182·1	182·1
Found (%)	89·9	88·4	90·1	89·56	91·6	89·6	92·2

The values for pyruvic acid computed according to the empirical factor of Fürth and Charnass give results accurate to within about $\pm$ 3 %.

The irregular results obtained when small amounts of pyruvic acid are estimated were at first supposed to be due to the length of time during which they were in contact with boiling acid, and unsuccessful attempts were made to find a time during which a maximum and constant yield was obtained.

In view of this irregularity, reduction at low temperatures was tried and the use of a zinc-copper couple in sulphuric acid solution was found satisfactory. The oxidation of lactic acid was carried out by a slight modification of a recent method due to Friedmann *et al.* [1927]. In this case the yield was lower than with other methods (about 80 %) but it was quite constant within a large range of pyruvic acid concentrations.

The apparatus used by us is shown in Fig. 1 and is much simpler than that of Friedmann. The transference of aldehyde to the receiver is facilitated by passing carbon dioxide from a cylinder through the apparatus. The gas is passed through a long column of saturated sodium bisulphite solution and then through water, which is frequently changed, before it enters the reaction vessel. The yield of aldehyde has been found to depend upon the rate of aeration also, and this can be effectively controlled.

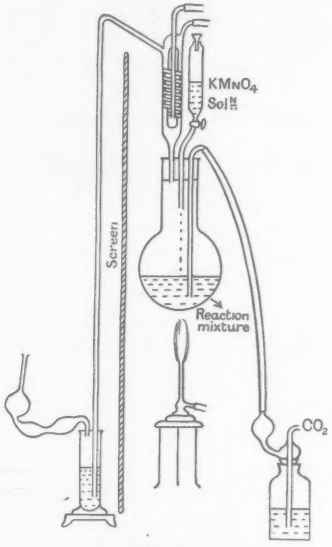

Fig. 1.

THE ESTIMATION OF PYRUVIC ACID IN PURE SOLUTIONS.

1 to 5 cc. of solution containing 0·25 to 15 mg. of pure pyruvic acid are treated with 50 cc. of 17·5 % H_2SO_4 and 0·5 to 1 g. zinc together with 1 cc. of 10 % copper sulphate solution. After an hour the solution is filtered into the reaction flask (a 500 cc. Kjeldahl flask) and neutralised slowly with 60 % NaOH added drop by drop, dimethylaminoazobenzene being the indicator. 10 cc. of 10 *N* H_2SO_4 containing 0·1 *N* $MnSO_4$ are then added and the oxidation is carried out with 0·01 or 0·005 *N* permanganate, the bound aldehyde being then titrated according to Clausen's method. The use of hydroxylamine to catch the acetaldehyde, as suggested by Leone and Tafuri [1925], was tried, but the end-point was not very sharp, and in all further work Clausen's method was adopted. Some of the results of estimations of pyruvic acid in pure solution with different quantities of the acid are given in Table III. A few experiments were also conducted in which the oxidation of the reduced

pyruvic acid was carried out with sulphuric acid. The yield of aldehyde obtained when 50 % sulphuric acid at 140° was used according to Clausen showed that more drastic treatment was required, namely increase of the temperature to 160°–170° and the use of 60 % acid, as had been found by Hill, Long and Lupton [1924] and Ronzoni and Laurence [1927]. Some of the results obtained are given in Table IV.

Table III. *Analyses of pyruvic acid in pure solutions.*

Pyruvic acid taken (mg.)	16·36	16·36	13·62	13·62	8·18	8·18	6·10	6·10
Found (%)*	99·6	99·4	101·9	97·8	99·3	99·3	100·6	100·6
Pyruvic acid used (mg.)	3·44	2·44	2·44	1·22	1·22	0·61	0·61	0·305
Found (%)*	97·7	97·6	97·6	102·3	98·4	103·2	100·0	100·0

* Using factor (see p. 1173).

Table IV. *Comparison of the permanganate and sulphuric acid methods of oxidation.*

Pyruvic acid taken (mg.)	8·43	8·43	5·06	3·37	0·61	0·305	1·69
Permanganate method, found (%)	80·4	82·3	80·3	78·9	80·3	82·6	76·3
Sulphuric acid method, found (%)	80·9	80·7	81·0	82·8	78·7	78·7	78·0

The results of the estimation of pyruvic acid in pure solution according to both the methods of oxidation, showed that there is a yield of 80 % aldehyde, constant over large variations in the quantities of pyruvic acid used. This does not appear to be due to a factor in the oxidation of lactic acid since pure zinc lactate solutions could be determined by the same method with an accuracy of 96·2 to 97·9 %.

The actual losses of pyruvic acid appear to occur during the initial reduction process, probably due to the formation, along with lactic acid, of products which do not yield bisulphite-binding compounds during the subsequent oxidation. Ronzoni and Laurence [1927], in connection with their experiments on the loss of lactic acid in solution, drew attention to an early observation of Dakin, who found that lactic acid solutions on standing yielded methylglyoxal. They moreover found that a solution of zinc lactate on standing for several days gave smaller and smaller yields of aldehyde day by day until after 4 days the yield stood constant at about 84 % for several weeks. This fall was noticed even when the solution was sterile and in the presence of mercuric chloride. We ourselves have observed such a loss of aldehyde, but the fall, though considerable, was not so rapid as that found by Ronzoni and Laurence. It was also found that zinc lactate solution, treated in the same way as pyruvic acid in our experiments, gave lower yields of aldehyde.

Another source of loss of lactic acid before oxidation appears to be due to the fact that it is kept in contact with sulphuric acid, and this loss increases with the time of contact. 23·3 mg. of zinc lactate were made up to 100 cc. with 20 % sulphuric acid. 20 cc. of this solution were taken at intervals of 1½ hours and the lactic acid was estimated. The results are given in Table V.

Table V.

Time during which lactic acid was in contact with sulphuric acid (hrs.)	0	1½	3	4½	6
Aldehyde yield (%)	97·6	96·5	95·4	94·2	93·1

Taking all these observations into consideration, we are led to imagine that the small yield of aldehyde in our case may be due to the same cause as that which lessened the yield of aldehyde with lactic acid solution on standing. The possibility exists of the formation of methylglyoxal from pyruvic acid if the carboxyl group is attacked, along with the carbonyl group, through the vigorous reducing action of the zinc-copper couple, although the formation of methylglyoxal is not known to occur from reduction of pyruvic acid in practice.

Since in the estimation of pyruvic acid by this method the errors due to reduction or oxidation are quite regular over a large range of concentration of pyruvic acid, the particular drawback of a small yield of aldehyde and the consequent necessity of using a large factor to compute the actual amount, cannot materially diminish the value of the method for the estimation of small quantities of pyruvic acid. This is shown by calculating the probable error of a single determination by the formula

$$e = \pm 0{\cdot}6745 \sqrt{\frac{d_1^2 + d_2^2 + \dots d_n^2}{n-1}},$$

where d represents the deviation of an observation from the mean of the series and n the number of observations. The probable error of the mean (e_m) is given by the equation

$$e_m = \frac{e}{\sqrt{n}}.$$

The probable error from a series of 15 determinations on the same quantity of pyruvic acid is shown in Table VI, and also the probable error of the mean.

Table VI.

10·18 mg. of pyruvic acid were reduced by 40 cc. of 17·5 % H_2SO_4 plus 0·75 g. Zn and a trace of copper sulphate.

Amount of acid taken (mg.)	Amount found (mg.)	Deviation from the mean (d)	d^2
10·18	8·10	−0·04	0·0016
	8·18	+0·04	0·0016
	8·04	−0·10	0·0100
	8·10	−0·04	0·0016
	8·03	−0·11	0·0121
	7·92	−0·22	0·0484
	8·27	+0·13	0·0169
	7·90	−0·24	0·0576
	8·06	−0·08	0·0064
	7·95	−0·19	0·0361
	8·14	+0·00	0·0000
	8·00	−0·14	0·0196
	8·29	+0·15	0·0225
	8·06	−0·08	0·0064
	8·16	+0·02	0·0004

Mean = 8·14
$\Sigma d^2 = 0{\cdot}2412$
therefore $e = 0{\cdot}0885$
and the error of mean (e_m) = 0·0237.

The probable error of a single determination is, therefore, about 1·1 %. The mean of this series is very nearly the same as that obtained from the determination with different quantities of pyruvic acid (Table III).

Thus, if an empirical factor covering the regular error be taken, the exact amount of pyruvic acid can be computed. This factor works out to about 20 %, *i.e.* 1 cc. *N*/10 iodine represents 5·5 mg. pyruvic acid. The values calculated on this empirical basis for various quantities of pyruvic acid have been given in Table III.

Application of the proposed method to biological fluids.

The question of the specificity of the reactions upon which the proposed method of estimation is based arises when the method is applied to biological fluids. It will be seen that not only hydroxy-acids, carbohydrates and acetone but also lactic acid and acetaldehyde interfere with the determination and must be removed from the solution before pyruvic acid is estimated. The separation of pyruvic acid from other bisulphite-binding compounds is not easy, and the results of titration cannot be taken as specific unless acetaldehyde from pyruvic acid only be taken as a measure of that acid. During the usual processes for the removal of carbohydrates or proteins from biological materials, these interfering agents are not removed. It is found that pyruvic acid bound by sodium bisulphite is not extractable by ether, whereas lactic acid and other hydroxy-acids are easily extracted. Therefore the pyruvic acid may be extracted along with lactic acid and other interfering substances by a preliminary ether extraction which separates them from carbohydrates also. The ether extract with a small quantity of sodium bisulphite solution is again extracted with ether, when the pyruvic acid, which is combined with bisulphite, is separated from lactic acid, β-hydroxybutyric acid and other interfering substances.

Thus, since ether extraction was used for the separation of pyruvic acid from carbohydrates and lactic acid, there was no necessity to adopt Van Slyke's method [1917] for the removal of carbohydrates from solution. It was moreover found that considerable amounts of pyruvic acid are lost in that process.

We had therefore to study only the separation of proteins from dilute pyruvic acid solution. Experiments were carried out with caseinogen and haemoglobin solutions to which small quantities of pyruvic acid were added. In the case of body fluids, it is found that the method of Folin and Wu [1919] can be employed. For the separation of proteins from yeast culture solutions, precipitation by alcohol is sometimes preferable. The solution is rendered faintly acidic and 15 to 20 times its volume of 99 % alcohol added. The solution is kept overnight, filtered, and the precipitate washed with 98 % alcohol.

An aliquot portion of the solution after the precipitation of proteins, either by the method of Folin and Wu or by the use of alcohol, is neutralised and

evaporated under reduced pressure at 40° to 50° almost to dryness. The residue is then transferred with a small quantity of saturated ammonium sulphate solution to the ether extractor, the solution rendered acid to methyl red, and the pyruvic acid extracted. The ether is evaporated and the pyruvic acid is bound by sodium bisulphite, and the solution is extracted again with ether to remove lactic acid, etc. It is estimated as before and the exact amount calculated by means of the empirical factor.

Table VII. *Recovery of pyruvic acid from protein solutions.*

Pyruvic acid added (mg.)	Protein solution	Method of precipitation	Pyruvic acid recovered (%)
4·61	2 % haemoglobin	Folin and Wu	99·2
4·61	5 % ,,	,, ,,	98·9
4·61	2 % ,,	Alcohol	99·35
4·61	5 % ,,	,,	98·9
7·69	3 % ,,	Folin and Wu	99·45
7·69	3 % ,,	Alcohol	100·1
15·37	5 % ,,	Folin and Wu	99·48
15·37	5 % ,,	Alcohol	97·8
3·05	2 % caseinogen	Folin and Wu	98·4
3·05	2 % ,,	Alcohol	97·7
6·10	3 % ,,	Folin and Wu	98·5
6·10	3 % ,,	Alcohol	100·2
15·25	3 % ,,	Folin and Wu	99·2
15·25	3 % ,,	Alcohol	98·3
15·25	5 % ,,	Folin and Wu	97·9
15·25	5 % ,,	Alcohol	97·6

From such dilute solutions of pyruvic acid a recovery of 97 % is therefore possible after protein precipitation.

The estimation of pyruvic acid in biological fluids may therefore be carried out as follows. The solution, which should contain not more than 15 mg. of pyruvic acid during its reduction to lactic acid, is taken and the proteins separated by either of the processes detailed above. Suppose the solution contains 0·05 %, about 2 to 5 cc. of this is used for protein precipitation and the whole of the filtrate is taken up for subsequent processes. The filtrate is rendered neutral to litmus and evaporated under diminished pressure at 40° to 50°. In this process much of the preformed acetone, acetaldehyde or other volatile compounds, escapes. The substance is then transferred to a Clausen or Meyerhof extractor with a small quantity of saturated ammonium sulphate solution, rendered slightly acid and extracted with ether to separate the carbohydrates from the ether-extractable substances. The ether should have been distilled over bisulphite, as it sometimes contains bisulphite-binding compounds as impurities. The ether extract is evaporated to dryness and shaken up with a quantity of sodium bisulphite three or four times in excess of the expected amount of pyruvic acid. It is then transferred again to the ether extractor to separate pyruvic acid from lactic acid, β-hydroxybutyric acid, phenols, etc. The residue is transferred to a 100 cc. flask and the pyruvic acid reduced by sulphuric acid and zinc with a trace of copper. The lactic acid is estimated as already described.

The errors introduced by the presence of acetone bodies is considerably diminished by the two evaporations. But even then the error due to acetone formed during oxidation, or held tenaciously, can be determined after the removal of aldehyde by Schaffer's method [1908], as adopted by Clausen as a correction for his lactic acid determination. The titre from this distillation is subtracted from the previous titre, and after proper corrections are applied the pyruvic acid is calculated using as factor 1 cc. of $N/100$ iodine = 0·55 mg. pyruvic acid. In many cases, except where the determination is carried out with yeast culture solutions, this determination of acetone bodies after final iodine titration is unnecessary, as the error is small. The results of the recovery of pyruvic acid added in small quantities to biological fluids, such as the body fluids of the lac insect, sheep's blood and yeast culture solutions, are given in Table VIII. The probable error of a single determination in a series, calculated from a number of experiments as before, is shown in Table IX.

Table VIII. *Recovery of pyruvic acid added to biological fluids.*

Medium	Amount added (mg.)	Iodine titre corrected for blank and titre (cc.)	Control (cc.)	Amount recovered using the empirical factor	Error (%)
Sheep's blood, 5 cc.	1·51	5·5 (0·005 *N*)	0·10?	1·49	−1·3
	3·02	11·3 (0·005 *N*)	0·10?	3·08	+2·0
	6·04	11·15 (0·01 *N*)	0·10?	6·08	+0·7
Yeast culture solution, 5 cc.	1·51	5·65 (0·005 *N*)	0·25	1·49	−1·3
	3·02	11·12 (0·005 *N*)	0·25	2·99	−1·0
	6·04	10·96 (0·01 *N*)	0·25	5·89	−2·5
Body fluid of lac insect, 5 cc.	1·51	5·76 (0·005 *N*)	0·20	1·53	+1·3
	3·02	11·02 (0·005 *N*)	0·20	2·98	−1·5
	6·04	10·86 (0·01 *N*)	0·20	5·87	−2·9

Table IX.

4·48 mg. of pyruvic acid were added to 5 cc. of the body fluid of the lac insect.

Exp. no.	Amount of pyruvic acid calculated (mg.)	d	d^2
1	4·44	+0·05	0·0025
2	4·30	−0·09	0·0081
3	4·38	−0·01	0·0001
4	4·51	+0·12	0·0144
5	4·35	−0·04	0·0016
6	4·32	−0·07	0·0049
7	4·27	−0·12	0·0144
8	4·56	+0·17	0·0289
9	4·46	+0·07	0·0049
10	4·36	−0·03	0·0009

Mean = 4·39.
Probable error $\epsilon = 0·063$; *i.e.* about 1·4 %.

Summary.

1. A new method for the estimation of pyruvic acid in small quantities has been described which is claimed to be more specific than the existing methods when applied to biological fluids.

2. The probable error of a single determination has been determined.

Our grateful thanks are due to Prof. R. V. Norris, for the keen interest he has shown in the work.

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CXLVII. THE SPECIFICITY OF THE DEHYDRASES.

THE SEPARATION OF THE CITRIC ACID DEHYDRASE FROM LIVER AND OF THE LACTIC ACID DEHYDRASE FROM YEAST.

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(*Received June 26th, 1928.*)

Since Thunberg [1920] showed by means of the methylene blue technique that tissues could oxidise a variety of substances, there has been a question as to the specificity of the systems involved. It seemed unnecessarily complicated to assume a separate enzyme for each acid oxidised. Yet a high degree of specificity is the characteristic of most enzymes, and it is therefore unjustified, until definite proof is available, to assume that one system is responsible for the oxidation of, say, citric and lactic acids. Thunberg believed that each substance oxidised had a specific enzyme, but this "is rather unconvincingly inferred from their unequal resistances to thermal changes" [Dakin, 1921].

In addition to this evidence, there is the extraction from muscle of the succinic acid dehydrase [Ohlsson, 1921], which has recently been separated from fumarase [Alwall, 1928]. Wishart [1923] extracted the succinic acid enzyme from liver, and Stephenson [1928] extracted the lactic acid dehydrase from bacteria. It has been shown [Bernheim, 1928] that the aldehyde oxidase in potato can reduce methylene blue and this enzyme can thus be classed as a dehydrase, as can the xanthine oxidase. Although the latter enzyme has been obtained chiefly from milk it is also possible to separate it from liver [Bernheim and Dixon, 1928] and from spleen [Morgan, 1926].

Investigation of the specificities of these four enzymes has shown that their action is almost entirely confined to their respective substrates. In the present paper the separation and properties of two further dehydrases are described, and these have also proved to be highly specific.

The citric acid dehydrase.

The oxidation of citric acid in the body has not been extensively studied. Battelli and Stern [1911] showed that the addition of citric acid to the liver increased the carbon dioxide output, and that this increase occurred just the same when the liver and the citrate were incubated *in vacuo* as when oxygen

was present. Meyerhof [1919] did a few experiments on the oxidation of citric acid by frog's muscle. The addition of methylene blue to the tissue and fumaric acid (which he regards as belonging to the same class as citric acid in respect to its oxidation by muscle) causes as much as a 30 % increase in the oxygen uptake. This may mean two things: (1) that the system reduces methylene blue more readily than it takes up oxygen, and the reoxidation of the methylene white gives the observed increase in the oxygen uptake, or (2) the system is unable to use oxygen directly but reduces a hydrogen acceptor in the tissue which is then reoxidised by oxygen. The increase of the oxygen uptake on addition of methylene blue would be due to a greater concentration of hydrogen acceptor being available.

The ability to utilise citric acid seems to be greater in herbivora than in carnivora, for Salant and Wise [1907] have shown that injected citrate disappears more quickly from the blood of the former than from that of the latter. Finally Amberg and McClure [1924] showed that about 100 mg. of citric acid were excreted in the urine every 24 hours.

In the following an enzyme has been separated from liver which will reduce methylene blue in the presence of sodium citrate but will not take up oxygen in a Barcroft apparatus. This is also true of the lactic acid dehydrase separated from *B. coli* by Stephenson, and from yeast in work described below. The product which citric acid yields has not been identified, but in the case of the lactic acid enzyme pyruvic acid is formed and has been definitely identified, so that there is no doubt that experiments carried out with methylene blue represent a true oxidation. In the intact animal, some of the citric acid may be directly oxidised to carbon dioxide and water, but there is some evidence that a part may be converted into sugars [Greenwald, 1915].

Preparation of the enzyme.

One pound of fresh liver, of pig, ox or sheep, is minced as finely as possible and treated four times with 300 cc. of acetone, each 300 cc. being filtered off with suction before the next is added. The resulting preparation is put in a desiccator and the acetone evaporated off *in vacuo*. The dried powder will then keep for several weeks in the air. To obtain a solution of the citric acid dehydrase, 30 g. of acetone liver are ground in a mortar with 100 cc. of water, and allowed to stand 2 to 3 hours at room temperature with intermittent grinding. The mixture is then squeezed through muslin and centrifuged, and the clear red solution is placed in a collodion sac and dialysed against distilled water for 6 or 7 hours. As the salts dialyse out a precipitate appears which after the dialysis is finished is centrifuged off. The clear solution containing haemoglobin is then used for the experiments.

To get rid of the haemoglobin the solution is half saturated with ammonium sulphate and the precipitate filtered off. The filtrate contains the haemoglobin but not the enzyme. The precipitate is washed with half saturated ammonium sulphate and filtered again. It is dried on the filter paper in a desiccator.

The dry powder thus obtained is dissolved in distilled water giving a clear light brown solution. The colour is due to traces of methaemoglobin that have been adsorbed on to the precipitate. The filtration in this process is slow, and the resulting enzyme has lost some of its activity. Therefore unless otherwise specified the solution containing the haemoglobin is used.

This solution will keep for several days in the ice chest. During this time a small amount of precipitate may settle out which is then centrifuged off without affecting the activity of the enzyme. This may also occur occasionally during an experiment when the solution is in a vacuum tube. The precipitate is probably a remnant of the protein which settles out during dialysis. The enzyme can be obtained in a powdered form from the haemoglobin solution by fully saturating with ammonium sulphate. The filtration of the resulting precipitate is very rapid and the precipitate can be dried in a desiccator. The powder gives a clear red solution with approximately the original activity of the enzyme. The activity of any given solution, however, depends on the liver used and the method of preparing the acetone liver. Quick treatment with acetone and thorough drying *in vacuo* is desirable.

If $M/15$ disodium hydrogen phosphate is used instead of water, the enzyme comes into solution in the same way but is accompanied by the succinic and xanthine dehydrases. None of the other common dehydrases is, however, present. When the clear centrifugate is dialysed a precipitate appears which is centrifuged off. The succinoxidase can thus be got into a clear solution whereas Ohlsson's preparation is turbid. The enzyme is not very active, and more work is necessary on the details of its preparation.

Other tissues have been tried as well as liver with similar results although the enzymes extracted were not very active. Ordinary muscle and the liver of embryo rats behave in the same way when treated with acetone and extracted with water or alkaline phosphate solution.

The specificity and properties of the enzyme.

Oxygen uptake of the enzyme. 3 cc. of a clear solution, an amount which will reduce 1 cc. of 1 : 5000 methylene blue solution in 20–30 minutes, were placed in a Barcroft apparatus and the oxygen uptake measured after 0·1 cc. of molar sodium citrate solution was added. No appreciable uptake occurred in 5 hours. The enzyme was then tested for its ability to reduce methylene blue, and it was found that the velocity of reduction had decreased, indicating a partial destruction of the enzyme due probably to the shaking in the air. This explains why the theoretical amount of oxygen is not taken up when methylene blue is added to the solution in the Barcroft apparatus.

The inability to take up oxygen by itself seems to be the property of the enzyme in the liver, at any rate under the conditions of the experiment. The acetone liver and the fresh liver from which the solution was made both reduce methylene blue more rapidly in the presence of citrate. Neither the acetone liver nor the fresh liver will take up more oxygen in the presence of citrate

than in its absence. The reason for this is not clear. Further work is being done on the general question of the oxygen uptake of the dehydrases.

Inorganic nitrate and *m*-dinitrobenzene [Lipschitz, 1921] were tried as possible hydrogen acceptors. The former was not reduced but the latter was. 0·1 g. of *m*-dinitrobenzene was added to solutions of enzyme with and without citrate. After incubation *in vacuo* at 37° for 2 hours, a few drops of soda were added to each. A deep purplish red colour developed immediately in the tube containing the citrate while a much lighter colour developed slowly in the control. Because of the original colour of the solution it was impossible to detect the yellow colour which develops before adding the soda.

The clear solution of the enzyme is specific for citric acid. None of the other possible hydrogen donators tried was oxidised. They included the following acids as sodium salts: succinic, malic, fumaric, lactic, tartaric, formic, glutamic, maleic, acetic, α-hydroxybutyric, oxalic, saccharic, and acetaldehyde. These were made up in 5 % solutions of which 0·1 cc. was used for each experiment. Thunberg vacuum tubes were used containing 3 cc. of the solution to be tested, 2 cc. of buffer, and 1 cc. of 1 : 5000 methylene blue. The time for complete reduction of the dye at 37° was noted.

The effect of aconitic acid was then tried. This is the unsaturated acid corresponding to citric acid. It is conceivable that under the influence of the enzyme it might add water to form citric acid which then could be utilised to reduce the methylene blue. Incubation of the enzyme with sodium aconitate showed that this was not the case. But because of the similarity of structure with citric acid, aconitic acid inhibits the reduction of methylene blue by the citric acid-enzyme system. The curve obtained seems to indicate that the aconitic acid is adsorbed on the enzyme surface so that part of the surface is unavailable for the citric acid. It is probable that increasing concentrations of aconitic acid act like increasing concentrations of citric acid, namely, in keeping the methylene blue from being accessible to the active centres. This will be discussed more fully below.

The effect of aconitic acid is specific, for the additions of acids like succinic, lactic or tartaric do not increase the reduction time at all. Fig. 1 shows the effect of varying amounts of aconitic acid on the reduction of methylene blue by the enzyme-citric acid system.

The reduction time of methylene blue was obtained when varying amounts of citric acid were present. In this experiment the haemoglobin-free solution was used. As is shown in Fig. 2 the activity, *i.e.* the reciprocal of the reduction time multiplied by 100, increases rapidly with increasing concentrations of citric acid and then falls off. The activity drops sharply from 0·01 cc. of 3*N* citric acid to 0·001 cc., *i.e.* *N*/200 and *N*/2000 respectively. *N*/2000 represents about twice the theoretical quantity of citric acid necessary to reduce the methylene blue present as calculated for two hydrogen atoms for every citric acid molecule. The rate thus falls off at a comparatively high concentration, namely, *N*/200.

The significance of this maximum is probably associated with the saturation of the enzyme surface with an amount of citric acid which enables an easy access of methylene blue, and yet is concentrated enough to ensure a quick

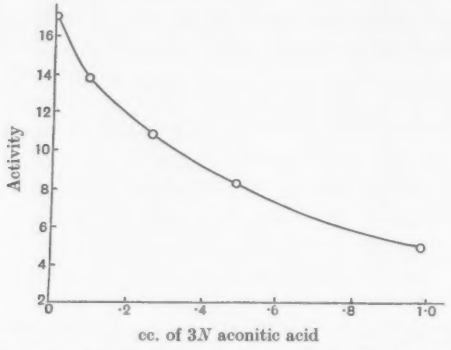

Fig. 1. Effect of aconitic acid on the reduction of methylene blue by citric acid.

reduction. When more citric acid is present the surface becomes supersaturated so that the methylene blue is no longer easily available. The inhibition is not a salt effect for equal amounts of *N* lactic acid or succinic acid do not inhibit. This inhibition at high concentrations of substrate has also been observed with the xanthine oxidase [Dixon and Thurlow, 1924]. In the following curve the concentration of citric acid is given in cc. per 6 cc.

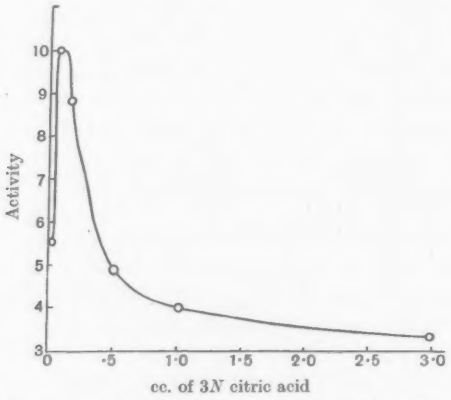

Fig. 2. Effect of varying concentrations of citric acid on the reduction of methylene blue.

The lactic acid dehydrase.

Lactic acid is not considered to be an intermediate product in the fermentation of sugars by yeast. It can, however, always be detected in yeast by extracting with acids or alkalis and applying the thiophen test to the extract. It must, therefore, play some part in the general metabolism. What its function is, is still doubtful. It is oxidised with difficulty. Fürth and Lieben [1922, 1, 2] found that a vigorous stream of oxygen through the yeast suspension was required before any appreciable oxidation occurred. They measured the oxidation by the increase of carbon dioxide produced when lactic acid was added to the suspension. The carbon dioxide produced was not however equivalent to the lactic acid present. They found that the lactic acid went partly into an ether-soluble substance and partly into the cell protoplasm. Hoffert [1926] showed that the lactic acid was not converted into unhydrolysable sugars or sugar-containing proteins. These workers have shown pyruvic acid to be present after allowing yeast to act on lactic acid. Harden and Norris [1915] showed that dried yeast plus lactic acid will readily reduce methylene blue and that pyruvic acid is quantitatively formed. This has been confirmed using untreated yeast and zymin (acetone yeast) by the following work, and is interesting in view of the observations of Fürth and Lieben that the addition of methylene blue made no appreciable difference to the amount of carbon dioxide arising from the oxidation of the lactic acid.

It is easy to show that whole yeast as well as dried yeast and zymin will reduce methylene blue more rapidly when lactic acid is present than without it, and that pyruvic acid is formed. It might therefore be expected that the pyruvic acid formed by the addition of methylene blue to the yeast lactic acid mixture would become decarboxylated and cause a rise in the carbon dioxide output. That Fürth and Lieben did not find this to be the case indicates the inability of the yeast to decarboxylate all the pyruvic acid formed.

But the following anomaly remains. Yeast contains a large amount of the lactic acid dehydrase as measured by the ability of yeast, dried yeast, or zymin to reduce methylene blue in the presence of lactic acid. For instance, 0·1 g. of washed zymin in 6 cc. of buffer, p_H 7·3, will reduce 1 cc. of a 1 : 5000 solution of methylene blue in 1 minute at room temperature. Without lactic acid no reduction occurs. The same is true for yeast. It is able to reduce methylene blue very quickly in the presence of lactic acid. Without the lactic acid the dye is reduced much more slowly. Yet despite this easy and rapid reduction of methylene blue, a vigorous stream of oxygen is necessary before any lactic acid is oxidised in the absence of the dye. This aeration seems a highly artificial condition which does not occur normally in the life of the yeast. The conclusion is that this dehydrase is unable to utilise oxygen and has very little to do with the oxidation of lactic acid unless methylene blue or some other hydrogen acceptor is present. The lactic acid must be oxidised by a system present in smaller concentration or less available so that vigorous aeration is necessary.

The inability of the dehydrase to utilise oxygen may be due to a lack of coenzyme to activate the oxygen. The existence of such a coenzyme seems indicated in muscle. In the presence of lactic acid washed muscle will reduce methylene blue. Szent-Györgyi [1925] has investigated the oxygen uptake of muscle in the presence of lactic acid. By extracting rat's muscle with ice-cold water he finds that the residue is able to reduce methylene blue with lactic acid, but unlike the unextracted muscle, it is unable to take up oxygen. This power is restored if the extract is added to the residue. Thus the extract contains some coenzyme which is able to activate oxygen so that it can be utilised by the lactic acid enzyme. The coenzyme is obviously unnecessary for the methylene blue which does not need activation.

In the following the lactic acid dehydrase has been separated from Delft baker's yeast. In the presence of methylene blue and lactic acid pyruvic acid is formed. This system will take up oxygen because the methylene blue is reduced to methylene white which then takes up oxygen on being reoxidised. No oxygen is taken up or pyruvic acid formed if methylene blue is absent. This fact confirms the inference that the enzyme in yeast is unable to utilise oxygen.

Preparation of the enzyme.

One pound of baker's yeast is broken up into small pieces and allowed to remain 8 to 10 hours exposed to the air. This allows water to evaporate and makes the treatment with acetone easier and more effective. It is then treated four times with 250 cc. of acetone, each portion being sucked off on a filter pump before the addition of the next. After the last treatment the zymin is dried as quickly as possible either with filter papers or in a vacuum. The resulting fine white powder will keep for months. It is important, however, that the zymin should be prepared quickly for a brown lumpy product does not yield a very active enzyme on extraction. 30 g. of the zymin are then ground up with 100 cc. of $M/15$ disodium hydrogen phosphate and allowed to stand from $3\frac{1}{2}$ to 4 hours at room temperature with intermittent grinding. The zymin is then centrifuged off and the solution which is almost clear is dialysed against distilled water for 6 or 7 hours until the solution in the bag gives a negative nitroprusside test for SH compounds. 3 cc. of the enzyme solution are then used for each experiment. The solution will keep in the ice chest for 24 hours but loses its activity rather quickly after that length of time.

If the extraction is continued much longer than 4 hours or if a stronger phosphate solution is used the succinic acid dehydrase which is present in yeast comes into solution with the lactic acid enzyme. The slightly cloudy solution gives only faint protein tests and only a slight precipitation when 2 % sulphosalicylic acid is added. The faint turbidity is not affected by filtering and the activity of the enzyme remains unchanged. Treatment with charcoal or kaolin resulting in a perfectly clear solution entirely inactivates the enzyme.

The cloudiness is increased when the solution is dialysed and disappears to a great extent when 2 cc. of buffer, p_H 7·3, are added to 3 cc. of the dialysed enzyme. The enzyme however is precipitated after standing for a short time at 37° with a solution of methylene blue. This fact may influence the rate of reduction of the dye to a small extent.

Specificity and properties of the enzyme.

The solution of the enzyme is specific for lactic and α-hydroxybutyric acids. None of the other possible hydrogen donators was activated. These included β-hydroxybutyric, malic, maleic, formic, citric, glutamic, succinic, fumaric, oxalic, acetic, tartaric, and pyruvic acids, and acetaldehyde and glucose. Glyceric acid which was also tried will be discussed later. Oxalic acid, however, has a marked inhibitory effect on the rate of reduction of methylene blue by the enzyme lactic acid system, as little as 0·1 cc. of a 5 % solution in 7 cc. causing an almost complete inhibition. That this is not a property of oxalic acid in general is shown by its entirely negative effect on the citric acid enzyme. Its molecule has in the juxtaposition of two CO groups a structure similar in an important respect to lactic acid, which may enable it to become adsorbed on the enzyme surface and thus keep the lactic acid from being activated.

The importance of this juxtaposition of the CO groups is shown by the fact that α-hydroxybutyric acid is oxidised whereas β-hydroxybutyric acid is not. The oxidation of α-hydroxybutyric acid is not quite as rapid as that of lactic acid. A solution of the enzyme which will reduce 1 cc. of a 1 : 5000 solution of methylene blue in the presence of 1 cc. of normal lactic acid in $5\frac{1}{2}$ minutes will reduce the same amount of methylene blue with 1 cc. of normal α-hydroxybutyric acid in 8 minutes. The presence of an extra carbon atom in the α-hydroxybutyric acid almost doubles the reduction time of methylene blue. β-hydroxybutyric acid when added to α-hydroxybutyric acid or to lactic acid does not increase the reduction time of the dye by the last two substances, showing that it is not adsorbed on the enzyme surface.

That pyruvic acid is the product of the action of the enzyme on lactic acid in the presence of methylene blue can be shown by adding to the reduced system a small amount of kaolin, shaking and filtering. The kaolin adsorbs the methylene blue and the clear colourless filtrate then gives the nitroprusside test for pyruvic acid. Pyruvic acid is not further oxidised by the enzyme in the presence of methylene blue so that additions of it to the lactic acid enzyme system ought to cause an inhibition. This is actually found to be the case (Fig. 3).

The inhibition, however, is not due entirely to the presence in excess of the product of the reaction and the consequent slowing of the velocity at which equilibrium is reached, because the pyruvic acid also inhibits the rate at which α-hydroxybutyric acid is oxidised. In this case it is not the product of the reaction. The most probable explanation is that pyruvic acid is adsorbed

on to the enzyme surface, and not being activated keeps the lactic and α-hydroxybutyric acids from being oxidised. This might be expected from the fact that oxalic acid is such a powerful poison and pyruvic acid resembles it in the juxtaposition of two CO groups.

Another example of this is glyceric acid. It is not oxidised by the lactic acid enzyme but inhibits the oxidation of lactic acid. Washed zymin is able to oxidise glyceric acid readily, and occasionally preparations of the separated enzyme will oxidise it slowly, indicating that there is a specific enzyme in yeast that will oxidise glyceric acid a part of which may be extracted with the lactic acid enzyme. The difference between the two enzymes is definitely proved by the fact that toluene does not affect the oxidation of the lactic acid at all, whereas the oxidation of the glyceric acid is markedly inhibited. The conclusion is that glyceric acid is adsorbed to a certain extent on the lactic acid enzyme but is not activated, thus causing an inhibition, and this is confirmed by the fact that enzyme extracts, which will not oxidise glyceric acid at all, are still inhibited by glyceric acid.

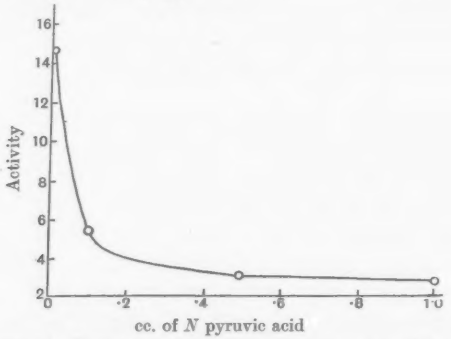

Fig. 3. Effect of pyruvic acid on the reduction time of methylene blue by lactic acid.

These results confirm those of Quastel and Wooldridge [1928] on the effect of various substances containing CO groups in juxtaposition on the oxidation of lactic acid by *B. coli*. Thus the idea that a molecule containing two contiguous CO groups is adsorbed on the enzyme and either inhibits the oxidation of the lactic acid or is oxidised itself seems valid. Exp. 1 shows the effect of glyceric acid on the oxidation of lactic acid, and Exp. 2 shows the effect of pyruvic acid on the oxidation of α-hydroxybutyric acid. Exp. 3 shows the effect of toluene on the oxidation of lactic and glyceric acids.

Exp. 1. 3 cc. of the isolated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of three tubes. Tube A also contained 1 cc. $N/2$ lactic acid and 1 cc. water, tube B 1 cc. $N/2$ lactic acid and 1 cc. $N/2$ glyceric acid, and tube C 1 cc. $N/2$ glyceric acid and 1 cc. water. The acids were added as sodium salts, and the experiment was carried out *in vacuo* at 37°.

Tube	Reduction time in minutes
A	8
B	17
C	40

Exp. 2. 3 cc. of separated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc. $N/2$ lactic acid and 1 cc. water, tube B 1 cc. $N/2$ lactic acid and 1 cc. $N/2$ pyruvic acid, tube C 1 cc. $N/2$ α-hydroxybutyric acid and 1 cc. of water, and tube D 1 cc. $N/2$ α-hydroxybutyric acid and 1 cc. $N/2$ pyruvic acid. The conditions were the same as in Exp. 1.

Tube	Reduction time in minutes
A	12
B	20
C	18
D	30

Exp. 3. 3 cc. of separated enzyme, 2 cc. of buffer, p_H 7·3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc. $N/2$ lactic acid, tube B the same plus 0·1 cc. toluene, tube C 1 cc. $N/2$ glyceric acid instead of the lactic acid, and tube D the same plus 0·1 cc. toluene. The conditions were the same as in the other experiments.

Tube	Reduction time in minutes
A	9
B	8
C	40
D	90

The rate of reduction of methylene blue was measured when varying quantities of lactic acid were present. The resulting curve (Fig. 4) differs markedly from that obtained for the citric acid enzyme in that increasing quantities of the substrate do not cause an inhibition. This is true both for lactic acid and for α-hydroxybutyric acid (Fig. 5). It might be possible to differentiate the dehydrases on this basis: on the one hand, the citric acid and xanthine enzymes which are inhibited by an excess of substrate, and on the other the lactic and succinic enzymes which are not. The curves for lactic and α-hydroxybutyric acids are similar. The velocity of reduction falls off in both cases when just under 0·5 cc. N acid is present. The figures are given in Figs. 4 and 5 as cc. in 7 cc. Consequently the velocity falls off when the concentration becomes less than $N/14$, a concentration which is far above the theoretical necessary to reduce the methylene blue present. In this respect too the difference from the citric acid enzyme should be noted, for the maximum concentration of substrate for the latter is about $N/200$.

Besides methylene blue oxygen was tried as a hydrogen acceptor. No uptake could be detected when the enzyme was shaken in a Barcroft apparatus in the presence of lactic acid. If methylene blue were added to the solution an oxygen uptake occurred because the dye was reduced by the enzyme-lactic

acid system and was reoxidised again by the oxygen present. In this case pyruvic acid can be shown to be present whereas no test can be obtained if the enzyme and lactic acid are shaken in oxygen without methylene blue. The enzyme suffers a certain loss of activity after being shaken in air for a length of time. As there is plenty of catalase present in the solution this loss cannot be due to a destruction by hydrogen peroxide. It is probably caused by a partial coagulation of the colloidal particles in the solution to form larger particles with less enzyme surface. An increasing turbidity is observed after shaking in air, suggesting that this may be the case.

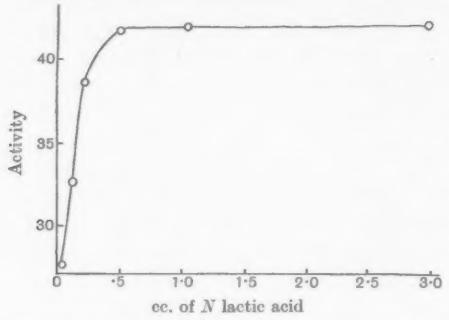

Fig. 4. Effect of varying concentrations of lactic acid on the reduction of methylene blue.

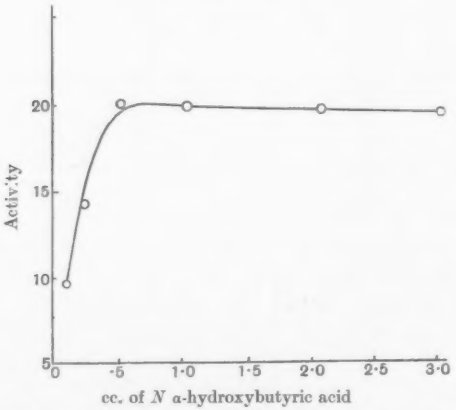

Fig. 5. Effect of varying amounts of α-hydroxybutyric acid on the reduction of methylene blue.

An attempt was made to supply active oxygen to the enzyme-lactic acid system on the possibility that pyruvic acid might be formed without the intervention of methylene blue. In order to do this a solution of xanthine oxidase, hypoxanthine, and a strong solution of peroxidase were added to the lactic acid enzyme and lactic acid. Oxygen was then bubbled through the

solution for several hours at 37°. The solution was then tested for pyruvic acid but with negative results. The peroxidase solution was made strong so that it could successfully compete with the catalase for the hydrogen peroxide.

Inorganic nitrate is not reduced. *m*-Dinitrobenzene gives a yellow colour when placed in a vacuum tube with the enzyme-lactic acid system, but this does not change to a purple on the addition of soda. If the reduction is allowed to go on for a long time, 5 or 6 hours, with a strong enzyme solution which will reduce 1 cc. of 1 : 5000 methylene blue in 10 minutes, a faint purple colour is seen on the addition of soda. It is obvious from the yellow colour produced that a reduction does take place but it seems to differ from the ordinary one found by Lipschitz for the dehydrases of tissue, the lactic acid one included. The possible explanation is that another reduction-product is formed along with traces of the usual nitrophenylhydroxylamine. These traces would account for the faint purple colour with soda, and the other reduction product for the deep yellow.

Discussion.

The work done on the reduction of methylene blue by tissues and bacteria is summarised in Table I. Frog's muscle (Thunberg) and *B. coli* [Quastel and Whetham, 1925; Quastel and Wooldridge, 1925] have been most extensively studied in regard to their ability to reduce methylene blue in the presence of various substances. Quastel and Wooldridge [1927] have treated *B. coli* in various ways showing that specific activations are destroyed by various treatments. These observations are very extensive and are not included in the table. The specificity of the alkaline phosphate extracts of washed muscle and liver is taken chiefly from Wishart. He found that a few substances such as formic acid and ketoglutaric acid were activated to a small extent by the extract, but the rates of reduction were so small compared to the rate with succinic acid that they are put as negative in the table. The data for the milk are taken from Dixon [1926], for the potato from Bernheim, and for dried yeast from Harden and Norris. Rat's muscle has been studied but not so completely (Bernheim and Dixon). The list of substances in the following table includes most of those that are active with either *B. coli* or frog's muscle. A + sign indicates that the substance will reduce methylene blue rapidly in the presence of the tissue or extract, a − sign that it will not reduce at a detectable rate. A blank space means that the substance still remains to be tested. Fumarase which is not a dehydrase has been included in the table.

From a consideration of the table there are three types of evidence for the specificity of the dehydrases. The first is the separation of the enzyme from the tissue. This evidence is fairly conclusive for the separation depends on differences in physical properties of the enzymes, *i.e.* the centres responsible for the activations are attached to different colloids. It would be difficult to explain these facts on the assumption of one enzyme in the tissue which was originally capable of effecting all the activations but which during the process of

Table I.

Tissue or extract	Acetaldehyde	Other aldehydes	Hypoxanthine	Alanine	Tryptophan	Histidine	Formic acid	Normal fatty acids to capronic*	iso-Valeric acid	Citric acid	Glutamic acid	Glyceric acid	Hydroxyglutaric acid	Lactic acid	α-Hydroxybutyric acid	β-Hydroxybutyric acid	Maleic acid	Malic acid	Succinic acid	Fumaric to malic acid	Tartaric acid	Glycollic acid	Certain hexoses and pentoses	Certain alcohols	Certain disaccharides
Washed frog's muscle	−	−	−	−	−	−	+	+	−	+	+	−	+	+	−	−	+	+	+	+	+	−			
Frog's muscle treated with liquid air ...	−	−	−	−	−	−	+	+	−	+	+	−	+	+	−	−	+	+	+	+	+	−			
Washed rat's muscle	−	−	−					+	−	+	+			+				+	+	+			−		
$M/14$ Na_2HPO_4 extract of washed ox's muscle	−	−	−	+				+	−	+	+			+				+	+	+	−				
Same heated ½ hour at 50°	−	−	−	−				+	−	+	+			+				+	+	+	−				
$M/14$ Na_2HPO_4 extract of washed ox's liver	+	+	+					+	−	+	+			+				+	+	+	−				
Water extract of acetone liver	−	−	−					+	−	+				+				+	+	+	−				
Resting *B. coli*	+		−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Water extract of autolysed *B. coli*	−	−	−	+			−	−				+		+	−	−		−	−		−		−		
Dried yeast (Lebedeff)	−	+		+			−	−				+		+	+				+			+			
$M/14$ Na_2HPO_4 extract of acetone yeast ...	−	−	−				−	−				+		+	+	−			+						
Milk	+	+	+	−	−	−	−	−		−	−			−			−		−				−	−	−
Potato	+	+	−					−		−							−	−	−		−		−	−	−

* Dixon (unpublished) has found that propionic acid is oxidised by rat's muscle and Thunberg (privately communicated) has found the same for frog's muscle. In his original paper Thunberg [1920] states that it is not oxidised.

extraction has been so altered as to appear specific for one substrate. Four dehydrases have been separated in this way: the succinic, lactic, citric and xanthine.

The second type of evidence is suggestive but not conclusive. It consists of treating the tissue in various ways, destroying certain activations and leaving others. Thus frog's muscle after treatment with liquid air will only activate succinic and hydroxyglutaric acids. The succinic acid dehydrase has by extraction been obtained free from the hydroxyglutaric acid enzyme, therefore it is justifiable to assume that the oxidation of the latter acid is due to a distinct enzyme. Then the water extract of autolysed bacteria will only oxidise lactic, glyceric and α-hydroxybutyric acids. As the original *B. coli* will oxidise β-hydroxybutyric acid this proves that there are two different enzymes responsible for the oxidation of α- and β-hydroxybutyric acids. Similarly zymin will oxidise lactic, glyceric and α-hydroxybutyric acids, but extracts can be obtained that will only oxidise lactic and α-hydroxybutyric acids, indicating that the oxidation of glyceric acid is due to a distinct enzyme. These arguments are not conclusive, however, until extracts are obtained which will oxidise β-hydroxybutyric or glyceric acid and not lactic and α-hydroxybutyric acids. Another type of argument is also possible. The enzyme from milk will oxidise purines and aldehydes, that from potato only aldehydes, and therefore the milk enzyme may be really composed of two specific entities. But as the xanthine oxidase from milk has been concentrated 4000 times and still retains the ability to oxidise aldehydes [Dixon and Kodama, 1926] it would be unsafe to assume this until further evidence is available. From these facts, then, the existence of three further specific dehydrases is indicated, namely, the enzymes that will oxidise hydroxyglutaric, β-hydroxybutyric and glyceric acids.

Summary.

1. Methods for the separation of the citric acid dehydrase from liver and the lactic acid dehydrase from yeast have been described.

2. The specificity and properties of these two enzymes have been studied.

3. The general question of the specificity of the dehydrases has been discussed.

My thanks are due to Sir F. G. Hopkins for his interest and encouragement, to Dr Malcolm Dixon for his advice and help, and to Dr J. H. Quastel for suggesting certain experiments.

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CXLVIII. SUCCINOXIDASE.

II. INFLUENCE OF PHOSPHATE AND OTHER FACTORS ON THE ACTION OF THE SUCCINODEHYDROGENASE AND THE FUMARASE OF LIVER AND MUSCLE.

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(*Received July 18th, 1928.*)

Muscle, liver and other tissues have been shown [Thunberg, 1909; Batelli and Stern, 1911] to contain a very powerful and remarkably specific enzyme, succinoxidase, which, according to Batelli and Stern, converts succinic into optically inactive malic acid. Einbeck [1914] regarded fumaric and not malic acid as the sole product, but later [1919] showed that the enzyme first converts succinic to fumaric acid and then, by a balanced reaction, to a mixture of fumaric (25 %) and malic (75 %) acids. Dakin [1922] showed that fumaric acid, reacting with muscle pulp, formed exclusively *laevo*-malic and not the inactive acid.

A number of attempts have been made to isolate the enzyme. Thus Batelli and Stern extracted it by means of dilute sodium hydroxide but obtained an enzyme of very low activity. Widmark [1918] improved the method by using 1·5 % sodium carbonate. Ohlsson [1921] substituted a $M/15$ solution of disodium hydrogen phosphate and this method has been generally used. Modifications, largely of the method of washing the muscle previous to extraction, have been introduced by Grönwall [1923], Svensson [1923], Sahlin [1924] and finally by Anderson [1927], who extracted the muscle several times with dilute saline before using $M/15$ phosphate, thus obtaining an enzyme extract considerably freer from proteins. All these investigators, however, have followed the course of the action of the enzyme in terms of the reduction times of methylene blue, using the usual Thunberg technique. Recently, Hahn and Haarmann [1927, 1928] and Fischer [1927] have shown that washed muscle, with sufficient methylene blue and sodium succinate, gives rise anaerobically to the same products, fumaric and malic acids, which it produces aerobically, and it seems likely therefore, that the enzyme system is the same in the two types of experiments.

In a recent paper [Clutterbuck, 1927] a polarimetric method was described for the determination of succinic acid in muscle and liver pulp in terms of its conversion into *l*-malic acid. Using this method, it was shown that liver was

eight times as active as the same moist weight of muscle in converting succinic or fumaric acid into malic acid, and cyanide, although completely inhibiting the oxidation or dehydrogenation of succinic acid, had no effect on the conversion of fumaric to malic acid. These experiments emphasised the fact that at least two enzymes were present in the oxidase system, a true succinodehydrogenase converting succinic to fumaric acid and a fumarase, converting fumaric to malic acid. This polarimetric method permits the study of both reactions and of the effect of various factors, other than cyanide, upon them. Also, by its means some preliminary experiments have been carried out, which are designed to discover the best method of separating the two enzymes from tissue and from one another.

Solubility and stability of succinodehydrogenase and fumarase.

One of the most striking differences, apart from the action of cyanide, between the fumarase and dehydrogenase is seen in respect to their solubilities and stabilities. The opalescent enzyme solution obtained by steeping washed rabbit's muscle in phosphate solution and centrifuging readily reduces methylene blue in presence of succinate and absence of oxygen and therefore contains the dehydrogenase. If, however, the solution is shaken a little by hand, the enzyme is completely inactivated. Moreover, when the phosphate enzyme solution or the aqueous extract of muscle is placed in a bottle with sodium succinate solution and rotated, the air being displaced by oxygen as in the experiments previously described [Clutterbuck, 1927], it was found that even the gentle rotational movement necessary to ensure oxygenation, although accompanied by only slight frothing, was sufficient to prevent any oxidation of succinate. During the rotation, however, a clot of coagulated protein formed, but the opalescence only slightly decreased. The clot probably contained the inactivated dehydrogenase, whilst the remaining turbidity was due to the fact that the fumarase protein complex under these conditions is quite stable, and coagulates only to a slight extent. A phosphate enzyme extract, rotated thus with sodium fumarate solution, showed in fact a six times greater fumarase activity than the original muscle pulp. Succinoxidase has also always been regarded as an extremely insoluble enzyme, only elutable by alkaline (phosphate, carbonate, etc.) solutions and after elution being readily precipitable by bringing the p_H of the solution to 6·5. It has been found, however, that although the dehydrogenase could not be detected in the aqueous extracts by our method, owing most probably to its inactivation during rotation, yet active fumarase is readily washed out of muscle by water or dilute (0·25 %) saline and is not precipitated to any great extent at p_H 6·5.

The "active" and "elutable" fumarase of muscle and liver pulp.

A number of experiments were therefore carried out in which the minced liver or muscle pulp (of rabbit) was steeped in an equal weight of water or 0·25 % saline, placed in a fine cloth and the fluid expressed. This process was

repeated 16 times and the extracts combined in fours and the activity determined polarimetrically. In order conveniently to compare the activity of the extracts, the production of 1·22 g. *l*-malic acid in 1 hour at 38° using 500 cc. of 1 % fumaric acid, as sodium salt, is called unit activity. The following results were obtained:

Muscle (150 g.)		Liver (50 g.)	
Saline or aqueous extracts	Fumarase activity units	Saline or aqueous extracts	Fumarase activity units
1–4	1·0	1–4	4·0
4–8	0·5	4–8	2·0
8–12	0·25	8–12	0·5
12–16	0·20	12–16	0·3

It will be seen that the amount of free or "active" enzyme, removed in the aqueous extracts of muscle and liver pulp, has approximately the same activity as the original pulp.

The muscle and liver residues were then steeped, on the one hand, in an equal weight of $M/15$ phosphate for an hour and, on the other, in an equal weight of $M/15$ bicarbonate solution to which a little carbonate had been added to bring the p_H approximately to that of the phosphate, namely about 9, and the extracts were then expressed as before. This process was repeated three times and the activity of each extract was determined. Finally the tissue residue was steeped twice for 24 hours and the activity of the extract found. Using phosphate as the eluting agent, expressing the results in units as above, we obtain the following results:

Muscle (150 g.)		Liver (50 g.)	
Phosphate extracts	Fumarase activity units	Phosphate extracts	Fumarase activity units
1 after 1 hour	4·0	1 after 1 hour	0·25
2 „ 1 „	2·0	2 „ 1 „	0·25
3 „ 1 „	0·6	3 „ 1 „	0·25
4 „ 24 hours	0·6	4 „ 24 hours	1·0
5 „ 24 „	0·5	5 „ 24 „	0·8

Using bicarbonate as eluting agent the results were similar but the activity was always somewhat smaller.

If we now convert the figures of our previous paper into units, whereas the fumarase activity of 100 g. muscle pulp was 1 unit, that of 100 g. liver pulp was about 8 units. Comparing these figures with those of the two sets of figures above, we obtain the following results.

(1) Liver pulp, weight for weight, has 8 or 10 times as great a fumarase activity as muscle pulp, but this only indicates that the liver pulp contains 8 or 10 times as much free or "active" enzyme as muscle.

(2) An amount of fumarase is removed from muscle and from liver by steeping 8 times in an equal weight of water or saline, approximately equal in activity in each case to the activity of the tissue pulp itself.

(3) After the amount of enzyme, extracted by this treatment with water or saline, has become reduced to a very small amount, steeping in $M/15$

phosphate or in a bicarbonate-carbonate buffer of the same p_H and expressing liberates from the muscle residue a further large amount of enzyme, having in the several extracts six times the activity of the original pulp, whereas similar treatment of the liver residue, the volume of which is however much smaller than the muscle residue, removes only a small amount of enzyme.

(4) The total activity of the water and phosphate extracts of 50 g. liver was approximately equal to the total activity of the extracts of 150 g. muscle.

It is concluded, therefore, that, although in the pulp liver appears 8 or 10 times as active as muscle in converting fumaric to malic acid, yet the total amount of enzyme, free and elutable, of liver is only about three times as much as in muscle, and, whereas almost the whole of the liver fumarase is free and active in the pulp emulsion, only about one-seventh of the total muscle fumarase is free and active, the remainder being readily eluted by alkaline bicarbonate-carbonate or phosphate buffer solution of p_H about 9. Borate buffer at this p_H gelatinised the muscle and no extract could be expressed. Washing the liver or muscle pulp with water or 0·25 % saline removes only the free, active enzyme, *i.e.* removes one-seventh of the total muscle enzyme but almost all of the liver enzyme. Previous workers, using washed muscle, have always employed either pulp in phosphate buffer or a phosphate enzyme extract of muscle and they have, therefore, by their technique, eluted and used in an activated condition that part of the enzyme not removed by washing.

The influence of phosphate on the action of the fumarase of muscle pulp.

That the activity of muscle fumarase is greater in phosphate than in bicarbonate buffer at the same p_H (7·2) is seen from the following experiment in which a sample of muscle (50 g.) was rotated, on the one hand, with fumarate (500 cc. 1 %) as control and, on the other, with fumarate to which had been added phosphate and bicarbonate sufficient to make the concentration 0·05 *M*, the p_H in all three cases being adjusted to 7·2. The following figures were obtained polarimetrically for the amount of *l*-malic acid produced.

Time (hours)	Control. *l*-Malic g.	+ Phosphate (0·05 *M*). *l*-Malic g.	+ Bicarbonate (0·05 *M*). *l*-Malic g.
0·5	0·45	1·30	0·60
1·0	0·78	2·22	1·05
1·5	1·26	2·66	1·61
2·0	1·55	2·90	1·97

It would appear therefore that phosphate, in addition to having the power (like bicarbonate) to elute the enzyme in virtue of its alkalinity, has also a direct activating effect on the fumarase. This activating effect was next investigated.

A large number of experiments with muscle pulp and fumarate have now been carried out and it has been found that the initial fumarase activity of the pulp varies considerably from animal to animal and often appears to be

related to the condition of the animal. In all the experiments recorded in this paper the usual apparatus was therefore duplicated, two bottles being rotated at the same speed in the same thermostat, one containing muscle and fumarate as control and the other muscle, fumarate and phosphate or other added substance. The usual 25 cc. sample was taken simultaneously from the two bottles, coagulated by heat and uranium acetate added. It was found that, if the flasks were then allowed to stand for half an hour, 75 cc. water being then added and allowed to stand for a further short period, the occasional difficulty previously reported of obtaining a clear filtrate for polarimetric observation never arose.

When phosphate is added to muscle and liver pulp and rotated with sodium fumarate solution, the rate of conversion to malate is greatly accelerated. That this is not a p_H effect, may be proved by adjusting the p_H of the two bottles to say 7·2 before the experiment when the same phosphate activation is still observed. Moreover, if a sample of the same pulp is caused to react with fumarate on the one hand in phosphate buffer, and on the other in borate buffer solution of the same p_H, whereas in the latter the rate is practically normal (there is actually a slight inhibition), in the phosphate buffer the usual acceleration is obtained. It would appear that the p_H optimum for fumarase covers a fairly wide range from about 6 to 9, and that changes of p_H over this range have relatively a slight effect on the rate of the reaction. The following results are typical examples obtained in a number of experiments using 50 g. minced sterile muscle pulp with 500 cc. 1 % fumaric acid (as sodium salt) rotating at 38° and comparing with a second similar sample of the same muscle and fumarate containing various amounts of added phosphate, the p_H in all cases being adjusted to 7·2. The figures give the *l*-malic acid (g.) produced, as determined polarimetrically.

Time (hours)	0·25	0·50	0·75	1·00	1·50	2·00
	l-Malic produced (g.)					
Control	0·31	0·67	0·97	1·32	1·82	—
+0·013 *M* phosphate	0·43	0·91	1·26	1·52	2·12	—
Increase of *l*-malic (g.)	0·12	0·24	0·29	0·30	0·30	—
Control	0·50	0·81	1·11	1·46	2·02	—
+0·026 *M* phosphate	0·63	1·14	1·65	2·02	2·64	—
Increase of *l*-malic (g.)	0·13	0·33	0·54	0·56	0·62	—
Control	0·34	0·61	0·90	1·14	1·55	2·02
+0·052 *M* phosphate	0·78	1·35	1·87	2·28	2·69	2·94
Increase of *l*-malic (g.)	0·44	0·74	0·97	1·14	1·14	0·92
Control	0·41	0·84	1·16	1·55	2·15	2·62
+0·078 *M* phosphate	0·47	1·03	1·41	2·11	2·82	3·22
Increase of *l*-malic (g.)	0·06	0·19	0·25	0·56	0·67	0·60
Control	0·40	0·80	1·15	1·50	2·10	—
+0·104 *M* phosphate	0·48	1·00	1·58	2·06	2·67	—
Increase of *l*-malic (g.)	0·08	0·20	0·43	0·56	0·57	—
Control	0·55	0·93	1·30	1·69	2·34	—
+0·156 *M* phosphate	0·61	1·22	2·00	2·62	3·29	—
Increase of *l*-malic (g.)	0·06	0·29	0·70	0·93	0·95	—

If the results of two of these experiments are graphed (Fig. 1), namely those with 0·052 *M* phosphate (which shows optimal activity) and with 0·156 *M* phosphate, it is seen that with the former, as also in experiments with smaller amounts of phosphate, the curve with phosphate (*A*) is regular and lies above the control (*B*), whereas with larger than optimal amounts (*C*, *D*)

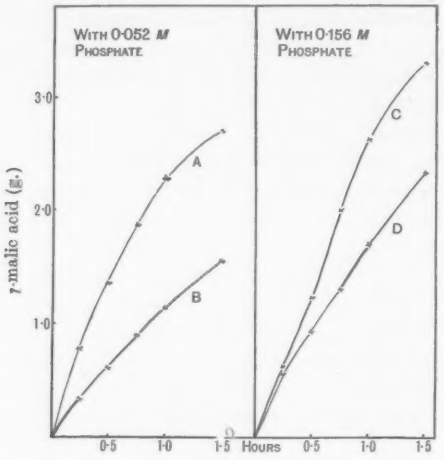


Fig. 1.

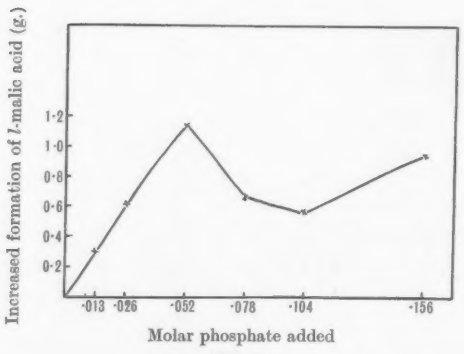


Fig. 2.

there appears at first an inhibition which is only overcome after about half an hour. The control curves for the muscle (*B*, *D*) of different amounts are all similar but vary considerably in steepness, and the activity in those muscles which have been examined in spring and summer appears on the whole to be higher than that of the muscle of animals previously recorded, which were examined in autumn and winter.

The effect of the phosphate concentration is best seen by plotting the concentration of added phosphate against the increased amount of *l*-malic acid found after 1·5 hours (Fig. 2).

From this curve it is seen that the increase in the formation of *l*-malic acid is almost directly proportional to the increase in phosphate concentration until the latter has increased to about 0·05 *M*. This appears to be an optimal concentration and with further increase the curve at first falls and then rises again. It is interesting to note that this optimum is identical with the phosphate optimum obtained by Platt and Dawson [1926] for the action of pancreatic lipase.

Effect of phosphate on the rate of dehydrogenation by muscle pulp.

Since the action of succinodehydrogenase is so very much slower than that of fumarase, it should be possible by carrying out similar experiments, with and without phosphate, replacing fumaric with succinic acid, to detect any effect of phosphate on the rate of dehydrogenation. Rotating 50 g. muscle in 500 cc. of 1 % succinic acid solution (as sodium salt) at 38° in an atmosphere of oxygen as control and a similar suspension containing phosphate (0·05 *M*), no influence on the rate of the dehydrogenation could be detected.

Influence of phosphate on the activity of liver pulp and of aqueous extracts of muscle and liver pulp.

The fumarase of liver pulp is activated by phosphate in the same way as that of muscle pulp. Thus, with one of the most active samples of liver that we have examined, the difference in the activity with and without the optimal amount of added phosphate was of the same order as in the muscle experiments; *e.g.* rotating 15 g. liver with 500 cc. of 1 % fumarate at 38° as control and a similar sample to which phosphate was added to make the concentration 0·05 *M*, the p_H being adjusted in both cases to 7·2, the following results were obtained.

Time (hours)	Control		+0·05 *M* phosphate		Increase of *l*-malic (g.)
	Rotation	*l*-Malic (g.)	Rotation	*l*-Malic (g.)	
0	0	0	0	0	0
0·25	1·02	1·14	1·44	1·61	0·47
0·50	1·74	1·95	2·44	2·73	0·78
0·75	2·10	2·35	3·07	3·44	1·09

Phosphate not only activates the fumarase of the tissue pulps but also activates the free eluted enzyme of the aqueous extracts of the pulps. Thus with 30 g. liver, eight aqueous extracts were combined, made up to 400 cc. and divided into two equal portions. One sample was rotated with fumarate, the final concentration being 1 % as before and the other with fumarate and sufficient phosphate to make the concentration optimal, the final p_H in both cases being 7·2. The results were as follows.

Time (hours)	Control		+ 0·05 *M* phosphate		Increase of *l*-malic (g.)
	Rotation	*l*-Malic (g.)	Rotation	*l*-Malic (g.)	
0	0	—	—	—	0
0·25	0·72	0·81	1·41	1·58	0·77
0·50	1·25	1·40	2·32	2·60	1·20
0·75	1·65	1·85	2·79	3·12	1·27
1·00	1·98	2·22	3·06	3·42	1·20

Similarly with the enzyme of aqueous extracts of muscle pulp, phosphate activates almost to the same extent as in the pulp experiments.

Influence of temperature on the fumarase activity of liver and muscle pulp.

The fumarase of liver, as we have already shown, is almost entirely in the free active condition and we might expect, therefore, to find that the activity of the enzyme, on rotating with fumarate at different temperatures, would change according to the well-known rule, namely, that for every rise of 10° the rate of reaction would be doubled or trebled. This was found to be the case. Thus with 15 g. of a very active sample of liver the following figures were obtained.

15 g. liver, 500 cc. fumarate, p_H 7·2, temp. 39°		15 g. liver, 500 cc. fumarate, p_H 7·2, temp. 19°	
Time (hours)	*l*-Malic formed (g.)	Time (hours)	*l*-Malic formed (g.)
0	0	0	0
0·25	1·00	1	0·96
0·50	1·80	2	1·67
0·75	2·46	3	2·29
1·00	2·84	4	2·83
1·50	3·40	5	3·24
2·00	3·44	6	3·44

The rate at 39° at the beginning of the experiment was almost exactly four times that at 19° but subsequently fell to three times.

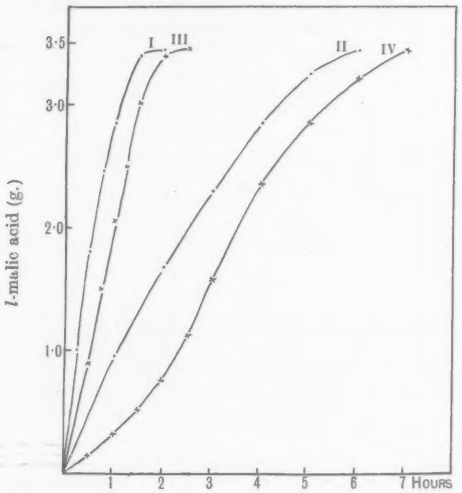
With a much less active sample of liver, the activity at 39° was five or six times that at 19° and on plotting the results (see Fig. 3) it is seen that with the less active sample at the lower temperature, there is a tendency for the curve to become more **S**-shaped. The figures for the less active liver are the following.

Time (hours)	*l*-Malic formed at p_H 7·2, temp. 39°	Time (hours)	*l*-Malic formed at p_H 7·2, temp. 19°
0	0	0	0
0·25	0·39	0·5	0·15
0·50	0·90	1·0	0·32
0·75	1·50	1·5	0·52
1·00	2·06	2·0	0·76
1·25	2·50	2·5	1·12
1·50	3·01	3·0	1·58
2·00	3·42	4·0	2·37
2·50	3·46	5·0	2·85
		6·0	3·22
		7·0	3·44

With muscle pulp, however, the influence of temperature appears to be somewhat different. We have already shown that only about one-seventh of the total elutable enzyme of muscle is free and active whilst the rest is adsorbed or in some way held in an inactive form but is elutable by phosphate or

bicarbonate-carbonate buffer of p_H 9·0. Using muscle pulp (50 g.) the activity as is seen from the accompanying figures is only twice as great at 39° as at 19°.

Time (hours)	*l*-Malic formed at p_H 7·2, temp. 39°	Time (hours)	*l*-Malic formed at p_H 7·2, temp. 19°
0	0	0	0
0·33	0·60	0·33	0·34
0·66	1·15	0·66	0·60
1·00	1·72	1·00	0·82
1·50	2·22	1·50	1·14
2·00	2·79	2·00	1·46
2·50	2·96	2·50	1·72



I. III. } Liver expts. at 39°. II. IV. } Liver expts. at 19°.

Fig. 3.

It is remarkable that the temperature coefficient with muscle pulp is so much smaller. Whether this is an adsorption effect or due to the production of some inhibitory substance at the higher temperature is not clear, but the former view is somewhat supported by the fact that if in place of the muscle pulp we use the aqueous extract of the muscle, then the coefficient is a little larger but even then it does not double every 10° as in the case of the liver enzyme. Thus, eight aqueous extracts of 100 g. muscle were combined, the p_H was adjusted to 7·2, the liquid made up to 800 cc. and divided into two equal portions and rotated with fumarate, one at 39° and the other at 19°. The results were as follows.

Time (hours)	*l*-Malic formed at 39° (g.)	*l*-Malic formed at 19° (g.)
0	—	—
0·33	0·47	0·21
0·66	0·86	0·32
1·00	1·28	0·47
1·50	1·50	0·60
2·00	1·79	0·87
2·50	2·22	0·94

At the end of the first hour the rate at 39° was roughly three times that at 19°.

The influence of other factors on fumarase and succinodehydrogenase activity.

Sodium chloride appears to inhibit very considerably the action of succinodehydrogenase, but only slightly the action of fumarase. Thus rotating 15 g. liver with 500 cc. 1 % succinate solution at 38° as control and a similar sample containing in addition 0·9 % sodium chloride, the following figures were obtained.

Time (hours)	Control. *l*-Malic (g.)	+0·9 % sodium chloride. *l*-Malic (g.)	Decrease of *l*-malic (g.)
0	—	—	—
2	0·50	0·22	0·28
4	1·25	0·38	0·87
5	1·60	0·52	1·08

Replacing succinate with fumarate, the inhibition of fumarase by 0·9 % sodium chloride is relatively very small.

Time (hours)	Control. *l*-Malic (g.)	+0·9 % sodium chloride. *l*-Malic (g.)	Decrease of *l*-malic (g.)
0	—	—	—
3	3·81	3·36	0·45

Sodium fluoride, on the other hand, has a well-marked inhibitory effect on fumarase. Thus rotating 50 g. muscle with 500 cc. 1 % fumarate at 38° as control and comparing with a similar sample containing also 1 % sodium fluoride, the following results were obtained.

Time (hours)	Control. *l*-Malic (g.)	+1 % sodium fluoride. *l*-Malic (g.)	Decrease of *l*-malic (g.)
0	—	—	—
0·25	0·28	0·10	0·18
0·50	0·50	0·22	0·28
0·75	0·67	0·29	0·38
1·00	0·91	0·46	0·45
1·50	1·25	0·78	0·47
2·00	1·52	1·08	0·44
3·00	2·11	1·64	0·47

Calcium chloride (4 %) caused a slight inhibition, and calcium nitrate a much greater inhibition, of fumarase activity.

Bile salts (2 %) caused a very slight acceleration.

Presence of fumarase in invertebrate muscle.

Pecten maximus. In his recent investigation on the chemical changes in muscle, Boyland [1928] found that succinic acid was present in extracts of the muscles of several invertebrates, particularly in *Pecten*. It seemed reasonable to expect, therefore, that the enzyme system would also be present. The large adductor muscles of several specimens of *Pecten* were minced, and 100 g. pulp rotated as before with fumarate at 17°. The following results show that fumarase is present in these muscles.

Time (hours)	*l*-Malic acid (g.)
0	—
1	0·16
2	0·38
3	0·50
4	0·63
5	0·77

Anodonta cygnea. The adductor muscles and the muscular part of the foot of a number of *Anodonta* were minced and 110 g. of the pulp rotated with fumarate at 18°. The muscle appeared about twice as active as the sample of pecten muscle examined above.

Time (hours)	*l*-Malic acid (g.)
0	—
1	0·26
2	0·59
3	0·98
4	1·37
5	1·77

Presence of fumarase in blood cells.

Defibrinated fresh ox-blood was centrifuged and the corpuscles and serum rotated with fumarate at 37°. The corpuscles showed a small activity but the serum did not appear to contain fumarase. The results with 80 g. corpuscles were as follows.

Time (hours)	*l*-Malic acid (g.)
0	—
0·5	0·07
1·0	0·11
2·0	0·16
3·0	0·24
4·5	0·44

Preliminary attempts at the isolation of fumarase.

Although liver appears to contain much more fumarase than muscle, the enzyme of the aqueous extract of liver is associated with such a large amount of protein, etc. that liver was regarded as an unsuitable source, and rabbit's muscle has been used in these experiments. The minced muscle (back and leg muscles) was first washed by steeping in an equal bulk of water and expressing 16–20 times. The free enzyme is lost in this process but a very large amount of protein, pigment, lactic acid, extractives, etc. are also got rid of. The washed muscle was then steeped for an hour in $M/15$ phosphate and the extract expressed. The process was repeated and the extracts combined.

When 1 % hydrochloric acid was added little by little to this phosphate extract, a precipitate was thrown down which began to coagulate when the p_H had fallen to 6·5. This precipitate contains the dehydrogenase and was filtered off. The precipitate and filtrate were then rotated in separate bottles with fumarate, the p_H being readjusted to 7·2 in both cases. The precipitate did not contain any fumarase but the filtrate had a powerful fumarase action. Alcohol and acetone added to phosphate extracts at the ordinary temperature gave inactive precipitates. When the phosphate extract is saturated with ammonium sulphate, however, a very active precipitate is obtained. This

precipitate may be dissolved in a little water and dialysed to remove most of the inorganic salts. As dialysis proceeds, some of the material reprecipitates. The contents of the sac were transferred to a measuring cylinder, the p_H adjusted to 7·2 and the material divided into two equal portions, one of which was rotated with fumarate and the other with fumarate and the optimal amount of phosphate. Not only did the control show very considerable fumarase activity but phosphate also showed the usual activating effect.

Similarity of glyoxalase and fumarase.

There is a close resemblance between the properties of fumarase, which adds on the elements of water to fumaric acid, and glyoxalase which adds on the elements of water to glyoxals, *e.g.* with methylglyoxal giving lactic acid.

Thus, glyoxalase was shown by Dakin and Dudley [1913, 1] to be sufficiently finely dispersed to be filtrable through paper without very great loss and fumarase appears to be similarly finely dispersed. Both enzymes are destroyed at some temperature above 48° but below 60°, and an active extract of both may be obtained by precipitating with ammonium sulphate, dissolving in water and dialysing. Both enzymes are eluted from rabbit's muscle by alkaline fluids and are more stable in alkaline solution. Moreover both enzymes are very widely and similarly distributed, the only difference in distribution so far observed being in the case of yeast. Yeast, according to Dakin and Dudley [1913, 1], contains glyoxalase but fresh samples (exposed for sale) on mixing with water and rotating with fumarate did not appear to contain fumarase. Finally the action of pancreatic juice, which contains antiglyoxalase, [Dakin and Dudley, 1913, 2] on the action of fumarase was investigated. The action of fumarase in an aqueous extract of muscle was inhibited but only to a very slight extent, which did not appear comparable with the inhibition of glyoxalase. This is being further investigated.

Summary.

1. The fumarase activity of rabbit's liver pulp is about eight times that of the same moist weight of muscle pulp but this must only be regarded as indicating that liver pulp contains eight times as much free or active enzyme as muscle.

2. An amount of fumarase is removed from muscle and from liver pulp by steeping eight times in an equal weight of water or 0·25 % saline approximately equal in activity in each case to the activity of the tissue pulp itself. Some succinodehydrogenase, which together with fumarase forms the succinoxidase system, is possibly also removed by washing but this could not be detected by the polarimetric method since the dehydrogenase becomes inactivated almost immediately by the gentle rotation necessary for oxygenation.

3. After the amount of enzyme extracted by this process of washing has become very small, steeping the tissue residue in $M/15$ phosphate or in a

bicarbonate-carbonate buffer solution of the same p_H (9) and expressing liberates from the muscle residue an amount of fumarase having about six times the activity of the original pulp, whilst from the liver residue only a small amount is obtained. The total fumarase activity of the aqueous and phosphate extracts of 50 g. rabbit's liver is approximately equal to the total activity of the corresponding extracts of 150 g. of rabbit's muscle.

4. Phosphate not only elutes fumarase in virtue of its alkalinity but also activates it, the optimal phosphate concentration at p_H 7·2 being 0·052 *M*. Phosphate does not appear to activate succinodehydrogenase in the same way as fumarase. Phosphate activates the fumarase of muscle and liver suspensions, of aqueous extracts of these and also of the enzyme preparation obtained by precipitating with ammonium sulphate and removing the salt by dialysis.

5. The temperature coefficient for the fumarase activity of liver pulp is normal (2–2·4) but of muscle pulp is very much lower (1·4). The activity of muscle pulp on reducing the temperature from 39° to 19° only falls to one-half. The coefficient for the activity of an aqueous extract of muscle is nearer normal.

6. Sodium chloride inhibits the action of succinodehydrogenase considerably, but of fumarase only slightly. Sodium fluoride inhibits the action of fumarase considerably.

7. The presence of fumarase has been detected in invertebrate muscle (*Pecten maximus*, *Anodonta cygnea*) but not in baker's yeast. It was present in the corpuscles but absent from the serum of fresh defibrinated blood.

8. A very active fumarase solution is obtained by precipitating the phosphate extract of washed muscle with ammonium sulphate, filtering and dialysing the solution of the precipitate.

In conclusion, the author desires to thank Prof. H. S. Raper for his interest, helpful suggestions and criticism throughout this work.

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CXLIX. A MICROMETHOD FOR THE DETERMINATION OF THE FATS AND LIPINS OF BLOOD.

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(*Received July 30th, 1928.*)

The chief micromethods which have been employed for the estimation of the fats and lipins are the oxidation method of Bang [1918] and Bang and Blix [1927], the nephelometric method [Bloor *et al.*, 1922], and the microtitration method [Stewart and White, 1925]. Bang's method has been recently modified by Bloor [1928]. A large number of papers has been published dealing with modifications of Bang's method. The chief references to the literature are given by Leites [1927].

The technique adopted in the following method is based on the principle of saponifying all compounds in the blood, which contain fatty acids, and of subsequently determining the amount of fatty acids isolated from the products of saponification. The advantages of this procedure are discussed fully by Leathes and Raper [1925], who also give references to the literature.

The original object of the following investigation was to apply Strebinger's [1919] method of oxidation to the estimation of the fats and lipins of blood. Strebinger has proved that the method gives accurate results with chemically pure substances; but, in the course of my own work, the method was found to be a laborious and exacting one when applied to the estimation of the fats and lipins in blood. All possibility of accidental contamination with any organic material such as fibres of filter paper must be excluded. Washed asbestos, or Jena glass tubes with filtering discs of fritted glass of porosity < 7 had to be used instead of paper. The method is therefore not well adapted for routine work.

A colorimetric method was therefore tried. This method is based on the formation of a coloured salt of the isolated fatty acids of the blood with the base of a dye-stuff. A number of basic dyes belonging to the triphenylmethane series was tried for this purpose. The base of malachite green has the advantage of being colourless in solution, while its salts are green, so that there is only one colour change involved in salt formation; but the interaction of the base with fatty acids is a slow process even when hastened by heat. Ultimately the base of Nile-blue hydrochloride, first used by Lorrain Smith [1908, 1911] for staining neutral fats and fatty acids, was found to give the best results. The colour change due to salt formation with fatty acid occurs rapidly.

Technique of method.

The Nile-blue hydrochloride used was extracted with ether and with xylene for 12 hours at room temperature. These solvents acquired only a faint red colour with yellow fluorescence; the oxazine dye therefore contained mere traces of the oxazone [Thorpe, 1907]. The 0·16 g. of Nile-blue hydrochloride was then dissolved in alcohol in a 500 cc. volumetric flask. Excess of silver oxide precipitated from 0·25 g. of silver nitrate by the addition of caustic alkali was washed alkali-free with water, dehydrated with absolute alcohol, and finally added as a suspension in alcohol to the solution of the dye-stuff. The solution was then made up to 500 cc. with redistilled absolute alcohol, thoroughly shaken and allowed to stand in the dark at room temperature until all the Nile-blue hydrochloride had been decomposed, and the solution had acquired the pure red colour of the free base. The solution was then filtered from silver chloride and excess of silver oxide through a Jena glass filter tube with fritted glass disc of porosity 5–7. When filtered through paper part of the dye is adsorbed, forming the blue coloured salt. The solution had a pure red colour, and no yellow fluorescence could be detected. A solution of suitable concentration to serve as an indicator for the estimation of the fatty acids isolated from about 0·1 cc. of blood was obtained by diluting part of this filtrate 1 in 10 with pure absolute alcohol. The diluted solution was stored in a brown bottle in the dark, and was found to retain its colour unaltered for several months, no yellow fluorescence being detectable. Since the molecular weight of Nile-blue A ($C_{20}H_{20}ON_3Cl$) is 353, the diluted alcoholic solution of the dye base is less than 0·0001 *M*.

Estimation of total fatty acid content of blood.

I. Bloor, Pelkan and Allen's method [1922] was adopted for the extraction of the fats and lipins of blood. 2 cc. portions of the alcohol-ether filtrate (= 0·1 cc. blood) were measured from a 10 cc. burette graduated in 0·02 cc. into test-tubes of about $\frac{3}{4}$ inch diameter. 0·5 cc. of 0·1 *N* alcoholic potash was added to each test-tube, the test-tubes were immersed in a beaker of water heated on an electric hot plate and the contents evaporated to dryness. The tubes were then placed in a drying oven at 80–85° for half an hour to remove traces of alcohol. The dry saponified residue was rendered acid with 0·1 cc. *M* phosphoric acid, care being taken in delivering the acid from a microburette that no drops were left adherent to the upper parts of the test-tubes. The tubes were then rotated so that the phosphoric acid was brought into contact with the whole surface of the dry residue, and were placed in a drying oven at 80–85° until the solution in each had been reduced in volume to a drop. 10 cc. of pure light petroleum of boiling-point 50–60° (redistilled from 30 % aqueous caustic potash) were added to extract the fatty acids and the solution was heated to the boiling-point, allowed to cool, and filtered through fat-free filter paper of 5·5 cm. diameter into test-tubes of accurately measured bore.

The tubes used were of resistance glass, and of 17 mm. bore as measured by a graduated cone reading to 0·1 mm. The acid residue was washed thrice with about 2 cc. portions of light petroleum, and the washings were filtered into these test-tubes. Care must be taken that none of the aqueous solution of mineral acid is transferred. The tubes containing the filtrates were then immersed in a beaker of water heated on an electric hot plate. The initial temperature of the bath was about 55° and was allowed to rise gradually to 75° in order to prevent loss of solution through bumping. When the greater part of the light petroleum had been driven off, the test-tubes were placed in a drying oven at 80° for about 30 minutes to remove the last traces of solvent. The tubes were then allowed to cool, and 10 cc. (measured from a microburette graduated in 0·02 cc.) of the dilute solution of the base of Nile-blue were added to each. The solutions were thoroughly shaken, and the tubes stoppered with corks previously washed with redistilled alcohol.

Preparation of standards for comparison. Pure palmitic acid (Kahlbaum), M.P. 62·5° (uncorrected), was recrystallised from light petroleum. 0·0641 g. of the recrystallised acid was placed in a 250 cc. flask, dissolved in light petroleum and made up to 250 cc., the solution being 0·001 *M*. Part of this solution was placed in a microburette with stoppered reservoir. The burettes used were either 2 cc., graduated in 0·01 cc., or 10 cc., graduated in 0·02 cc. Seven numbered test-tubes of uniform bore (17 mm.) were placed in a stand, 0·48 cc. of 0·001 *M* palmitic acid was measured into test-tube (1), and then successively (2) 0·58, (3) 0·69, (4) 0·83, (5) 1·0, (6) 1·20, (7) 1·44 cc. into the other test-tubes, the quantities being arranged in a geometrical progression with a common ratio of 1·20. Palmitic acid being a weak acid only converts a fraction of the dye base into the blue coloured cations of the salt. If necessary, the series of tubes may be extended to nine with a progressive increase in the amount of the blue coloured fraction of the dye. The solutions were evaporated to dryness in the water-bath, the last traces of light petroleum being removed by heating the tubes in a drying oven at 80°. 10 cc. of the alcoholic solution of the Nile-blue base were next added to each tube. The test-tubes were then closed with corks previously washed with redistilled alcohol, and sealed with paraffin wax. Their contents were thoroughly mixed and the solutions allowed to stand for ten minutes. If kept in the dark the standard solutions retain their colours unaltered for several days. The colours of the solutions in the test-tubes containing the fatty acids isolated from blood were then compared with those of the standard series. The following concrete example may be given. The solution of the fatty acids isolated from 0·1 cc. of blood was found to have a colour intermediate between tubes (4) and (5) of the standard series. 0·1 cc. blood therefore contained more than 0·00083 and less than 0·001 millimol. fatty acid. An approximate estimate of the fatty acid in the blood was thus obtained with an error of less than 20 %. A more accurate result may be obtained by preparing another standard series of tubes with quantities of palmitic acid in a geometrical progression with a smaller common ratio such

as 1·05 covering the range between 0·00083 and 0·001 millimol. A more convenient but somewhat less accurate method is to add the alcoholic solution of Nile-blue base from a 10 cc. microburette to the tube containing the fatty acid from blood until the colour of the solution in it matches with no. 4 of the standard series. If y be the total volume in cc. of the diluted solution, then the quantity of fatty acid in it is $\frac{0{\cdot}00083 \times y}{10}$ millimol. The results of the analyses given later are stated as millimol. fatty acid per 100 cc. or, in the case of those obtained by Bang's procedure, per 100 g. blood. The results may also be stated in terms of any of the higher fatty acids, palmitic, oleic, or stearic, by multiplying the result in millimols. by the factor 0·256, 0·282 or 0·284. The total fatty acid content of 100 cc. of defibrinated ox blood was thus found to be 0·83 (4 estimations), 0·82 (3 estimations), 0·80 (2 estimations) millimol. of fatty acid. When Bloor's method is applied to whole blood, Bloor [1928] states that the extraction of fats and lipins may not be quite complete. Hence the foregoing results are possibly too low, but are in good agreement with one another in view of the small quantities of blood analysed.

II. In a second series of experiments Bang's method of weighing the blood for analysis was adopted. About 100 mg. (98·5 to 126) of defibrinated ox or sheep blood were placed on a weighed slip of fat-free absorbent paper, and the weight of blood determined by difference, a torsion balance reading to 1·0 mg. being used. The pieces of paper with absorbed blood were then dried in the air, placed in numbered test-tubes and stored in an evacuated desiccator over calcium chloride.

1. *Determination of the total fatty acid content of the blood.*

This estimation, which gives the total fatty acids present as neutral fats, soaps and lipins, was carried out in the following way. The weighed slips of paper with dried blood were extracted with 10 cc. of boiling absolute alcohol. The tubes containing the alcoholic extracts were allowed to stand overnight. The alcoholic solutions were again boiled, cooled and transferred to a second set of test-tubes. The paper slips with blood were then washed once with alcohol and twice with ethyl ether, and the washings transferred to the second series of tubes. 0·5 cc. 0·1 N alcoholic potash was added to each, the tubes were immersed in water in a beaker, heated on an electric hot plate, and their contents evaporated to dryness. The saponified material was then heated in an electric drying oven at 85° for at least 30 minutes to remove the residual alcohol. The higher fatty acids were set free by the addition of 0·1 cc. of M phosphoric acid to the saponified residue. The other details of the procedure were the same as those already described in the method for analysing the alcohol-ether filtrate used in Bloor's method.

In order to ensure that all solvents and papers used were free from fats, a blank experiment was carried out with a negative result, the red colour of the alcoholic solution of the Nile-blue base remaining unaltered.

2. *Estimation of fatty acids derived from neutral fats.*

The neutral fats were extracted from the dried blood with light petroleum. Bang [1918] obtained low values for the neutral fat by the oxidation method, varying from 0·01 to 0·05 % in human blood of normal individuals on a mixed diet. For this reason larger amounts of blood were used. The first estimation was carried out with 0·211 g. of ox blood absorbed on two slips of fat-free paper. For the two estimations of neutral fat in sheep's blood, the quantities taken for analysis were 0·363 g. and 0·337 g., the blood samples being absorbed on three slips of paper. The dried blood was boiled in each case with 10 cc. of light petroleum, and the extracts were allowed to stand at room temperature in stoppered tubes for at least 12 hours. The extracts were filtered, and evaporated to dryness. The residues were then saponified with 0·5 cc. of 0·1 *N* alcoholic caustic potash, and 3 cc. of alcohol. The remaining details of the technique were the same as those already described for the estimation of the total fatty acids. Since the concentration of fatty acids isolated from the neutral fat of blood is normally much lower than that of any of the standard solutions of palmitic acid, the quantity *y* cc. of the dilute alcoholic solution of Nile-blue base finally added to obtain matching of the colours was always much less than 10 cc.

In view of the possibility that the low values for fatty acid of neutral fat might be due to incomplete extraction by the light petroleum, the following method of successive extraction with alcohol and light petroleum was tried. The samples of dried blood were first extracted with absolute alcohol, and the residues left after evaporation of the alcohol were extracted with light petroleum. The light petroleum extract was then filtered, and treated in the way already described. The values given in the table are much higher than those obtained by direct extraction with light petroleum. A preliminary extraction with alcohol thus appears to set free part of the neutral fat, or to render it accessible to the solvent action of light petroleum.

A few experiments were also carried out with acetone as solvent. The acetone was allowed to act on the dried blood at room temperature for 24 hours.

Some of the values obtained by the foregoing method are recorded in the following table, the same sample of ox or sheep blood being used for all the determinations.

Millimol. fatty acid per 100 *g. blood.*

Blood	Alcoholic extract	Light petroleum (direct extraction)	Alcohol and light petroleum extraction	Acetone
Ox	0·83	0·07	0·42	0·49
	0·81	—	0·43	—
	0·82	—	—	—
Sheep	0·88	0·12	0·45	0·52
	0·90	0·12	0·43	0·53
	0·91	—	0·44	0·50
	0·88	—	0·43	—

Purification of solvents.

All solvents must be completely freed from acids and fats. Light petroleum (B.P. 50–60°) ("analytical reagent and free from aromatic hydrocarbons") was thoroughly washed with a concentrated aqueous solution of potassium hydroxide, and distilled from this solution in an all-glass still. The absolute alcohol was redistilled from calcium oxide. The ethyl ether was washed with an aqueous solution of caustic alkali, freed from aldehyde and acids with solid potassium hydroxide and finely powdered potassium permanganate, decanted into a second bottle and dehydrated with calcium chloride. It was finally distilled. The acetone was washed for several days with a saturated solution of potassium carbonate, and distilled. All distillations were carried out in all-glass stills, with ground in glass connections.

Each solvent was then tested in the following way. About 20 cc. were evaporated to dryness in a test-tube, and 10 cc. of the alcoholic solution of Nile-blue base were added. The red colour of the solution of the base remained unaltered. This simple method enables one to ascertain whether any given solvent can be used with safety.

All glassware was washed with a solution of chromic acid in concentrated sulphuric acid. Washing with potassium permanganate solution (about 2 %), followed by the removal of any adherent manganese dioxide with hydrochloric acid, was found later to be an equally effective and more convenient method [Horst, 1921].

Discussion.

The present communication deals only with the technical details of the method. Any discussion of the significance of the experimental figures would be premature, until a much larger number of comparative analyses has been completed. The writer intends to apply the method to the investigation of human blood.

Summary.

1. A method for the estimation of the total fatty acids in small quantities of blood has been described.

2. By selective extraction of the dried blood with different solvents, the fatty acid content of some of the components in the mixture of substances extractable by alcohol can be determined.

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CL. PROTEIN AND VITAMIN B.

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(*Received July 12th, 1928.*)

Both protein and vitamin B[1] are accepted as essential constituents of the diet, but the amount of protein which can be metabolised without harmful results is a question on which there is a variety of opinions. For some time it was thought that a high protein diet produced nephritis, but more recent work seems to show that an animal can tolerate large proportions of protein in its diet. Osborne and Mendel [1924] succeeded in feeding rats on a diet containing as much as 80 % caseinogen, while Osborne, Mendel, Park and Winternitz [1926] gave a diet consisting of 95 % caseinogen and found that the rats appeared fit and well. These observers obtained good growth to adult size with rats on high protein diets, but noticed hypertrophy of the kidneys, when the protein comprised one-third or more of the total ration.

Addis, MacKay and MacKay [1926] find that rats can "live for a third of their life-span on a diet very high in casein (70 %) or with added cystine, and yet escape any damage to their kidneys." Jackson and Riggs [1926] fed rats on diets containing 76 % caseinogen, and 20 % caseinogen + 56 % egg-albumin; they found hypertrophy of the kidneys, but no nephritis. The success of these various workers in feeding high protein diets suggests that the failure of others with similar diets is due, not to the high proportion of protein itself, but rather to a deficiency of some other dietary factor or to a lack of balance between some of the constituents. There is evidence that vitamin B is closely related with protein metabolism.

Hartwell [1922, 1, 2, 1924, 1925, 2] has shown that there is a definite relation between the amount of vitamin B and protein in the diet of the lactating rat. Reader and Drummond [1925] found that rats fed on a diet containing 20 % caseinogen grew to adult size, and attained a weight of over 300 g., but when the protein was raised to 45 % none of the rats reached 250 g., while on 90 % of caseinogen no rat grew to 150 g. The animals appeared to be in good health, and *post mortem* examinations showed nothing abnormal, except hypertrophy of the kidneys. In a later paper [1926] these observers confirmed this result, and further found that in order to obtain good growth on such a diet the proportion of yeast extract (vitamin B) must be raised so

[1] By the term vitamin B is meant the original water-soluble B which has now been shown to consist of at least two factors.

that "the ratio of protein to yeast has a value 5 or under." Thus there is evidence that for maximum growth the vitamin B of the diet must be increased when the protein is increased. The proteins used in experiments quoted above were caseinogen, and a mixture of caseinogen and egg-albumin. Bond [1922] obtained good growth in rats on a diet containing 30 % of egg-albumin and 5 % marmite. This relation between protein *qua* protein and vitamin B suggests that there may be some proteins which will require more vitamin B than others for their metabolism, since proteins vary considerably in their composition. Experiments to be described in this paper demonstrate that for normal metabolism edestin requires far more vitamin B than does caseinogen or egg-albumin.

Experimental.

Piebald rats bred in the laboratory were used. Twelve rats, 6 ♀ and 6 ♂, were used in each experiment, except where otherwise stated, and each experiment was started when the rats weighed approximately 40 g., except Exp. 11. The basal diet consisted of

20 g. edestin[1].
64 g. potato starch.
12 g. butter.
4 g. salt mixture [Hartwell, 1922, 1].
300 cc. distilled water.

The food was made freshly every day except Sunday, the "cooked" method being used [Hartwell, 1925, 1]. The same sample of marmite was used throughout. The rats were allowed food *ad lib.*, and distilled water to drink. They were weighed daily for the first 4 weeks, and afterwards the weighing was omitted on Saturdays and Sundays. The animals were not kept on screens (except for part of Exp. 9) because this work was started in 1925 when the necessity for such a precaution was not realised. The floor of the cage was covered with sawdust to which the animals had access. All the experiments to be described in this paper were made under the same conditions and are therefore comparable. The growth curves are given in Fig. 1.

Exp. 1. Basal diet + 2 g. marmite.

After about a week several of the rats became weak and were obviously ill, 5 of them died on the 13th day, having shown a previous loss in weight. The remaining 7 were killed and *post mortem* examination showed only one to be normal, while the other 6 had kidneys of a deep purple colour and gorged with blood. (The histological details will be described in a later paper.)

Exp. 2. Basal diet + 2 g. marmite.

The results described above were so striking that it was thought advisable to repeat the experiment. Accordingly 12 more young rats were fed on a similar diet, and 6 died by the 14th day; 4 more were killed and each showed the typical purple kidneys. The remaining 2 were kept for 9 weeks on the diet

[1] The edestin used in these experiments was made by Messrs E. T. Pearson and Co. Ltd.

and were then killed. Their kidneys were slightly yellowish, but otherwise normal.

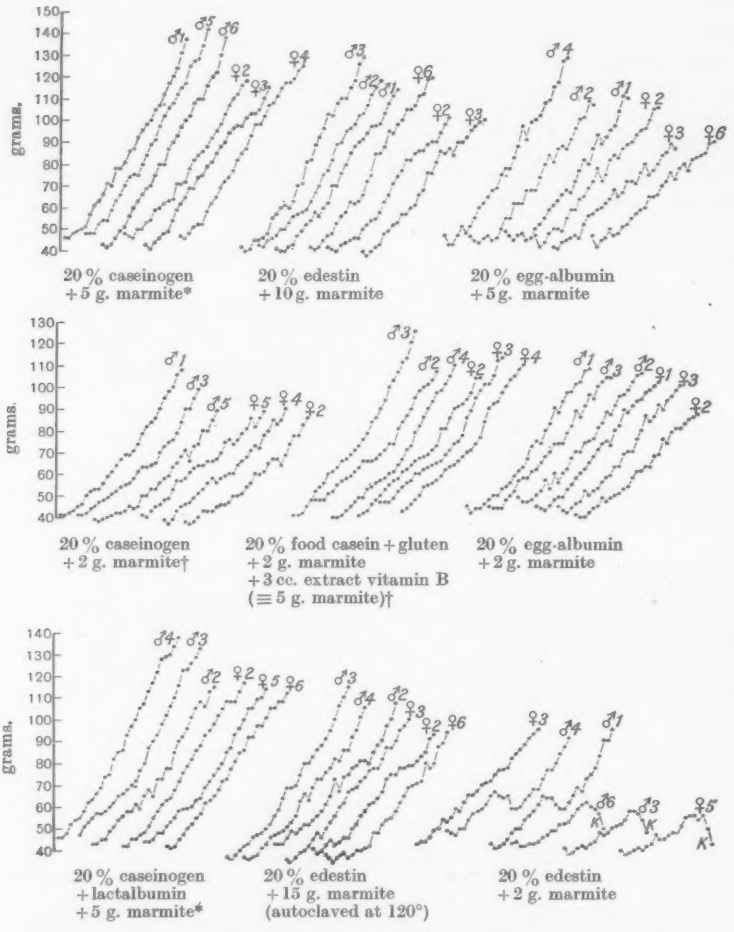
grams.
20 % caseinogen +5 g. marmite*
20 % edestin +10 g. marmite
20 % egg-albumin +5 g. marmite
20 % caseinogen +2 g. marmite†
20 % food casein + gluten +2 g. marmite +3 cc. extract vitamin B (≡ 5 g. marmite)†
20 % egg-albumin +2 g. marmite
20 % caseinogen +lactalbumin +5 g. marmite*
20 % edestin +15 g. marmite (autoclaved at 120°)
20 % edestin +2 g. marmite
Days

Fig. 1. Growth curves of rats on synthetic diets.
* Diets described by Hartwell [1927].
† " " " [1926].
K Rat killed.

Exp. 3. Basal diet + 2 g. ma.mite.

It was thought that the above results might be due to some alkaloid adhering to the edestin, and therefore a new sample was prepared by the

method of Osborne and Mendel [1894]. The results appeared little better, only 2 rats out of 12 showing normal kidneys after 5 weeks on the diet. Three animals died in 14 days and one was killed after a bad loss in weight; this rat had the typical purple kidneys.

Exp. 4. Basal diet + 2 g. marmite.

In the next experiment the edestin used was subjected to several hours' further washing in cold water, in the hopes of removing the hypothetical impurity. Two rats died in 14 days and 4 others which showed the typical loss in weight were killed; all 4 had purple kidneys. After 4 weeks on the diet, the remaining 6 were killed and 5 appeared to be normal. These results were certainly better, but as half the rats developed abnormal kidneys, the edestin could still not be regarded as a satisfactory food protein. It was therefore decided to try edestin which had been extracted with alcohol and if that failed to try extraction with ether.

Exp. 5. Basal diet + 2 g. marmite.

The edestin was extracted with 93·58 % ethyl alcohol by weight, the extraction being repeatedly carried on for a period of 3 weeks.

Of the 12 rats used in this experiment, 1 died within a week and 2 more on the 18th day, at which time 4 others which showed loss of weight were killed; all 7 showed the typical purple kidneys. The remaining 5 animals were killed after 5 weeks on the diet, and only one had normal kidneys.

Exp. 6. Basal diet + 2 g. marmite.

The edestin used in this experiment was extracted with alcohol as in Exp. 5 and then further extracted with ether repeatedly for a period of 2 weeks.

After 2 weeks on the diet 1 rat was eaten, and therefore 5 others which had lost weight were killed; each had enlarged and purple kidneys. The other 7 rats were kept for $3\frac{1}{2}$ weeks on the diet and then killed; macroscopically 5 appeared to have normal kidneys.

Exp. 7. Basal diet + 2 g. marmite + 3 cc. extract containing vitamin B [Hartwell, 1922, 2].

In view of these results, it seemed unlikely that any poisonous substance adhering to the protein was responsible for the bad effects and therefore it was thought advisable to alter the proportions of other constituents of the diet. To the basal diet + 2 g. marmite were added 3 cc. of an extract containing vitamin B (≡ 3 g. marmite). On this diet 7 rats died in 14 days, at the end of which time the other 5 were killed, only 2 of them showing normal kidneys.

Exp. 8. Basal diet + 15 g. marmite.

18 rats were used in this experiment and 15 g. marmite were given. With this addition to the diet, the growth curves were good and no loss in weight occurred. Six rats, 3 ♀ and 3 ♂ were killed after 2, $3\frac{1}{2}$ and 5 weeks respectively on the diet, and each animal had normal kidneys, thus it was obvious that the yeast extract had prevented the bad symptoms.

Exp. 9. Basal diet + 15 g. marmite (modified after 4 weeks).

Exp. 8 lasted only 5 weeks and it was decided to repeat it and continue

the diet for a longer period in order to see if the improvement were temporary or permanent, since the addition of marmite might have delayed, but not prevented, the kidney symptoms. The basal diet + 15 g. marmite was given for 4 weeks and after this the rats were placed on screens in order that the work might be comparable with another series of experiments. At the same time the basal diet was modified so that the mineral salts were increased, 8 g. instead of 4 g. being used, while the starch was correspondingly lowered to 60 g. instead of 64 g. This modification was introduced because there was some indication in another experiment that the proportion 4 % of mineral salts was rather low for breeding. On this modified diet the rats lived for 12 weeks. All the animals were in excellent condition, their fur was thick and silky and did not come out at any period. The modification in diet and technique introduced at the end of the 4th week made no appreciable difference to the growth curve. During the experiment 5 does each produced 2 litters and the 6th one had 3 foetuses in the uterus when killed. *Post mortem* examination showed that all organs and tissues were macroscopically normal, except for the thyroids which were slightly enlarged.

Exp. 10. Basal diet + 10 g. marmite.

An experiment was next made using only 10 g. marmite to see if this quantity were adequate. After 4 weeks on the diet all 12 rats appeared fit and well and their growth curves were good, in fact the animals grew at the same rate as when they received 15 g. marmite. Accordingly 2 ♀ and 2 ♂ were killed and as *post mortem* examination revealed no abnormalities, the other rats were not killed.

Exp. 11. Basal diet + 2 g. marmite (older rats).

The next problem was to see if rats of all ages developed kidney symptoms on the edestin diet and therefore an experiment was made using partly grown rats. Six males ranging from 98 to 145 g. and 5 females from 85 to 110 g. were used. From weaning until given the edestin diet, the rats were treated as stock animals, *i.e.* they received a little bread and milk every day and kitchen scraps *ad lib.* After 14 days on the edestin diet, 2 ♀ and 2 ♂ were killed; their kidneys appeared quite normal. After 6 weeks, 2 ♀ and 2 ♂ were killed; and as they also were normal in all respects, the experiment was discontinued. One doe produced a litter of 6 and reared 4 of them (but the marmite was increased during the lactation period). All the rats grew steadily and well, though less rapidly than on a control diet of kitchen scraps.

Exp. 12. Basal diet + 15 g. marmite autoclaved at 120° for 4½–5 hours.

(*a*) This diet was given for 17 days during which time the rats grew well and appeared to be in good condition. Since the loss in weight and death of some animals was usually noticed before the 14th day, it seemed that these rats were safely over the danger period, but 4 were killed to see if the kidneys were abnormal; all 4 however appeared quite normal.

(*b*) A second experiment was made in which the rats were kept on the basal diet + autoclaved marmite for 6 weeks. During this period the growth

curves were good and steady, no loss in weight being shown. The rats were all in excellent condition at the end of the 6 weeks, therefore the factor in marmite which prevented the kidney symptoms had not been destroyed by the high temperature.

Previous experiments with other proteins have shown good growth with 5 g. or less of marmite in 100 g. dry solid food. Hartwell [1926] obtained steady growth in rats on a diet containing 20 % caseinogen and only 2 g. marmite, and quite good growth with the equivalent of 5 g. marmite and a mixture of "food casein" and gluten. Later [Hartwell, 1927] caseinogen + egg-albumin, and caseinogen + lactalbumin produced satisfactory growth when 5 g. marmite were added to 100 g. solid. Thus it appeared that the metabolism of edestin required more marmite than other proteins and in order to test this hypothesis further, experiments were made with synthetic diets containing 20 % of egg-albumin with 5 g. and 2 g. marmite respectively.

Exp. 13. Basal diet in which edestin was replaced by egg-albumin + 5 g. marmite.

Twelve rats were fed on this diet for 4 weeks; growth was good and the animals were fit and well throughout the experiment. *Post mortem* examination of 3 ♀ and 3 ♂ showed normal kidneys.

Exp. 14. Basal diet as in Exp. 13 + 2 g. marmite.

In this experiment 6 ♀ and 5 ♂ rats were used; they grew well during 4 weeks on the diet. Then 2 ♀ and 2 ♂ were killed, but nothing abnormal was seen.

Results.

1. *Edestin experiments.*

A synthetic diet containing 20 % of edestin as the sole protein and 5 g. or less of marmite per 100 g. solid is not a satisfactory food for young rats. Many of the animals die during the first 2 weeks of the experiment, with a characteristic drop in weight before death. Other rats show a similar depression of the weight curve (Fig. 1), but eventually recover. The animals which die become limp and cold, with sunken eyes, but otherwise no external symptoms can be detected. There is no paralysis as with a vitamin B-free diet. *Post mortem* examinations were made on 84 rats and of these only 18 showed normal kidneys. The typical depression in the weight curves is indicative of abnormal kidneys. Partly grown rats can live on a diet of 20 % edestin and 2 g. marmite, and do not develop kidney symptoms.

2. *Amount of yeast extract in relation to kind of protein.*

With egg-albumin, the addition of 5 g. marmite instead of 2 g. per 100 g. dry solid made no appreciable difference in the growth curves of the rats, but a corresponding alteration in the caseinogen diet led to a marked improvement in the rate of growth. With edestin on the other hand, 10 g. of marmite were needed to provide for good growth.

3. *Rate of growth.*

When egg-albumin was used as the sole protein, the rate of growth was just slower than with either of the other proteins, but the curves were quite good, and the rate of growth steady. With edestin, the growth was equal to that obtained with caseinogen, provided a larger proportion of marmite were added to the basal diet. There appeared to be no difference in the rate of growth of the rats on the edestin diet when they were given 15 g. or 10 g. yeast extract, but with the autoclaved marmite (15 g.) the rate of growth was a little slower.

COMMENTS.

It seems unlikely that the edestin in itself is responsible for the bad effects obtained in these experiments, because this protein has been used successfully by other nutrition workers for a considerable time. Osborne and Mendel [1916] fed rats on a diet containing 15 % of edestin for 11 weeks, and in other experiments [1919] they used 17·9 % of edestin for a period of 8 weeks. Osborne, Mendel, Park and Winternitz [1926] obtained good growth to adult size with 16 % of edestin, while Osborne and Mendel [1924] were successful in feeding rats on a diet containing as much as 50–55 % edestin. This latter work is very interesting in that Osborne and Mendel state "several attempts to raise animals on fat-free diets containing 95 % of protein of which 90 % was edestin failed because the animals died during the preliminary period of adjustment to the food." Their rats obviously died in a short time, as did mine on the edestin diet, so it is possible that this failure is due to insufficient vitamin B in the diet, especially as they fed 80 % caseinogen successfully. It is not possible to make any definite statement, because there is no way of comparing the respective amounts of vitamin B used, since their yeast (of which 400 mg. were given as a daily ration) may have been relatively stronger or weaker in vitamin B than the marmite used by me.

Exp. 9, in which the rats lived for 16 weeks on a diet containing 20 % of edestin as sole protein, also implies that edestin is a satisfactory constituent, especially as breeding took place at the normal time. It is, however, clear from the experiments described in this paper that for good growth edestin requires more marmite than either caseinogen or egg-albumin. It is possible that some specific amino-acid present to a greater extent in edestin than in other proteins used for feeding experiments is responsible for the amount of marmite required. That there are obvious differences in the metabolism of amino-acids has been shown by Seth and Luck [1925] who found that in the case of histidine there is no increase in the urea content of blood even after 6 hours, while other amino-acids cause appreciable urea production. At the present time one cannot make more than a tentative suggestion on this point, because the analyses of proteins in respect of amino-acids vary so considerably. Since marmite is used in synthetic diets solely as a source of vitamin B, it

can be assumed that more vitamin B is required for the metabolism of edestin, and also, as marmite autoclaved at 120° for 4½–5 hours was equally satisfactory in preventing the kidney symptoms, the antineuritic substance [Chick and Roscoe, 1927] is obviously not the important factor in this case. Presumably the rats obtained sufficient antineuritic substance from excreta to which they had access, or from some constituents of the diet (the butter, or protein which was not pure). It is extraordinary that the older rats did well when only 2 g. marmite were given, and that no kidney abnormalities resulted, especially in view of the work of Osborne and Mendel [1922] demonstrating that the amount of vitamin B required by the rat is proportional to the weight and age of the animal. No doubt when more is known of the complex nature of vitamin B, these discrepancies will disappear, for we are dealing with several factors, not yet completely separated. From the experiments described in this paper the kidney symptoms are undoubtedly characteristic of young rats, possibly analogous to the development of rickets, which disease is only met in young animals.

These experiments support the view that there is a definite relation between the protein and vitamin B of the diet, and further suggest that different proteins may require various amounts of vitamin B in order that they may be properly metabolised. Egg-albumin appears to require less vitamin B than the other proteins used. This may be correlated with the fact that egg-albumin provides for slower growth and therefore a lower proportion of marmite is adequate.

SUMMARY.

1. On a synthetic diet containing 20 % of edestin and 5 g. yeast extract per 100 g. dry solid, many young rats die, showing typical kidney abnormality; older rats are not adversely affected by the diet.

2. These symptoms can be prevented by the addition of more yeast extract to the diet.

3. The "protective" factor in yeast extract is not destroyed by autoclaving at 120° for 4½–5 hours.

4. Edestin requires more yeast extract than either caseinogen or egg-albumin for normal metabolism in young growing rats.

I wish to thank Mr Clark and Mr Faraday of Messrs E. T. Pearson and Co. Ltd. for their help and co-operation in this work, and also Miss W. M. Clifford for making the *post mortem* examinations.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CLI. THE MINIMUM AMOUNT OF VITAMIN D REQUIRED FOR A POSITIVE ANTIRACHITIC EFFECT IN THE "LINE" TEST.

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(*Received July 30th, 1928.*)

Fosbinder, Daniells and Steenbock [1928] made an attempt to determine quantitatively the amount of energy necessary to secure a demonstrable deposition of calcium in the bones of a rachitic rat. Using a monochromator and taking infinite pains to measure the energy transmitted from the radiation of 265$\mu\mu$ wave-length from a mercury vapour lamp to a "pure" sample of cholesterol, they found that the shortest time of this treatment needed to produce enough vitamin D to give what they term a positive antirachitic action in their "line" test, was 22·5 seconds. The energy absorbed by the "cholesterol" during this time was 234 ergs. The number of quanta absorbed was calculated as $3{\cdot}2 \times 10^{13}$. Applying Einstein's law of photochemistry, this would imply that $3{\cdot}2 \times 10^{13}$ molecules of vitamin D had been synthesised during the exposure of 22·5 seconds. The number of gram-molecules is then 5×10^{-11} and assuming the molecular weight of the antirachitic material to be essentially the same as that of cholesterol (385) the weight of vitamin D generated in this experiment was $5 \times 10^{-11} \times 385 = 2 \times 10^{-8}$ g. (It should be noted that this was the whole dose during the 10 days' test period, not a daily dose.) The American workers then prophesied that when pure vitamin D was obtainable this would be found to be approximately the amount required to give a positive result in the "line" test for the antirachitic factor. The writer has obtained results with a sample of irradiated ergosterol which appear to prove this prophecy to have been singularly correct.

The solution of irradiated ergosterol used in the Society's laboratory as a standard preparation of vitamin D was prepared by Mr T. A. Webster at the National Institute for Medical Research under conditions calculated to generate the maximum antirachitic activity in it. Its potency was determined by testing on rats rendered rachitic by the method of Steenbock and Black [1925]. The writer had had the privilege of 10 months' experience of this test under Prof. Steenbock's tuition, so that her standards and assays may fairly be considered comparable with his.

The result of the assay may be summarised thus:

Daily dose of irradiated ergosterol mg.	Degree of calcification in Steenbock's terms of assay
0·0	—
0·000005	Few foci
0·00001	+ (a thin line)
0·00002	+ + (a thicker line)
0·00005	+ + + (a thicker line still)
0·0001	+ + + + (complete healing)

Where Steenbock classified degrees of calcification as "negative," "positive," and "very positive," as he has done in his latest paper, his "positive" result may be considered equal to a + or + + of the above classification, preferably the latter.

The daily dose that gave this positive result was 2×10^{-5} mg., so that the amount consumed in 10 days was 2×10^{-7} g.

Rosenheim and Webster [1927] state that maximum antirachitic activity is generated in ergosterol by their method of irradiation after 30 minutes and that at this time they can recover 90 % of unchanged ergosterol. Assuming that some of the vitamin D formed during the first 30 minutes of irradiation had been destroyed by the rays that appear to keep formation and destruction balanced during the subsequent 180 minutes, it would appear that rather less than 10 % of the irradiated ergosterol consisted of vitamin D. The amount of actual vitamin D contained in 2×10^{-7} g. of the Society's standard preparation of irradiated ergosterol would according to this be about 2×10^{-8} g. This gives an extraordinarily clear confirmation of the American workers' prophecy that the actual amount of vitamin D required to give a positive reaction in the "line" test would prove to be 2×10^{-8} g. Incidentally, also, there is evidence here of the suitability of the "line" test for demonstrating the presence of even smaller quantities than this of vitamin D.

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CLII. NOTES ON THE IRRADIATION OF ERGOSTEROL.

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(*Received September 1st, 1928.*)

It was recorded by Rosenheim and Webster [1927] that the exposure of ergosterol to ultra-violet radiation produced a mixture of substances including vitamin D, and evidence was given suggesting that, for moderate periods of irradiation, the amount of vitamin present in the mixture was nearly independent of the period of irradiation, apparently owing to simultaneous formation and destruction of the vitamin. This work has been continued in search of possible means of isolating vitamin D.

I. *The effect of using "filtered" light at various temperatures.*

Attempts were made to produce more concentrated preparations of vitamin D by the irradiation of ergosterol by selected portions only of the ultra-violet spectrum. If, as was suggested by Morton, Heilbron and Kamm [1927, 1] and also by Pohl [1926], vitamin D shows absorption chiefly between 230 and 260$\mu\mu$, while ergosterol shows it chiefly between 260 and 290$\mu\mu$, then the use of a filter cutting off all rays of wave-length shorter than 265$\mu\mu$ should increase the quantity of vitamin D formed during irradiation by reducing the rate of destruction of the vitamin without seriously affecting that of ergosterol. The use of "vitaglass" for this purpose was suggested by Morton *et al.* in the above paper, and has since been tested by them [1927, 2], and by Rosenheim and Webster, with negative results. Alcoholic cobalt chloride is, however, even more suitable for such a test. A 5 % solution of thickness 10 mm. in a cell with plane polished silica walls, transmits between 20 % and 30 % of radiations between 400$\mu\mu$ and 280$\mu\mu$ and about 17 % of 280$\mu\mu$. The transmission then falls rapidly to 3 % at 265$\mu\mu$ and then very rapidly indeed to less than one part in a million at 254$\mu\mu$. By multiplying the exposures used without a filter by about 4·5, the use of this filter thus secures an undiminished exposure to radiation above 280$\mu\mu$ and only slight diminution of radiation from 280 to 270$\mu\mu$, with freedom from any disturbance at all by radiation of wave-lengths shorter than 265$\mu\mu$. Preliminary trials with this filter showed no effect on the

equilibrium, and, as it seemed possible that temperature changes during radiation were affecting the results, the trials were extended over a wide range of temperature.

Experimental details.

The ergosterol was a highly purified crystalline specimen of M.P. 162° prepared from ergot, presented to Dr Rosenheim by Dr H. D. Jowett of Messrs Burroughs Wellcome and Co. and kindly placed at our disposal by Dr Rosenheim. The solvents used were pure ether dried over sodium, and absolute alcohol, these being chosen as the most stable solvents available without marked ultra-violet absorption. Solutions were freshly made before use, and in these experiments were always of concentration 0·1 g. per litre.

For all except the lowest temperatures the source of light was a K.B.B. silica mercury arc worked at atmospheric pressure with 2·5 amp. and 125 volts across the arc. An opaque screen with a circular hole 5 cm. diameter was placed 5 cm. from the lamp and 10 cm. from the ergosterol solution. This solution was contained in a fused silica cell 10 mm. thick, with sides 2·5 × 2·5 cm. of optically plane polished silica. It was immersed in an outer vessel of fused silica containing a fluid kept at constant temperature and a thermometer. For the tests at + 1° and − 18° an air chamber dried by calcium chloride and fitted with a silica window was attached to the side of the bath facing the lamp to avoid deposition of dew. At each temperature exposures were made both to unfiltered radiation and to radiation filtered through the cobalt chloride filter described above. (This filter was circular in section and 2 inches in diameter.) A uniform exposure of 20 minutes for unfiltered light and 90 minutes for filtered light was given in order to secure equal amounts of radiation of wave-lengths between 280 and 350$\mu\mu$.

For the tests made at very low temperatures the above cell was immersed in liquid oxygen or nitrogen and a Hewittic vacuum silica mercury arc was placed horizontally 10 inches above the cell, and run at 3 amp. and 130–140 volts after a preliminary test with a photoelectric cell to show that these conditions gave radiation of intensity roughly similar to that used at other temperatures. No part of the body of the cell was above the surface of the liquid, and no exposure to ultra-violet radiation occurred before complete cooling. Alcoholic solutions only were used at these temperatures since they froze to a transparent glass without separation of solute, in contrast to ethereal solutions which solidified as an opaque crystalline mass. The silica cell was occasionally broken by a sudden shattering of the contents, due apparently to further contraction of the glassy mass after it had become rigid. Unfiltered light only was used, and exposure varied from 5 minutes to 5½ hours. All solutions were photographed through a Hilger quartz spectrograph before and after irradiation.

The following table shows the temperatures used and the fluid surrounding the cell at each temperature.

+ 77·8°	Alcohol (boiling)
+ 30·6°	Ether (boiling)
+ 1°	Water
− 18°	Ether (boiling at low pressure
−183° approx.	Liquid oxygen
−195° „	„ nitrogen

Biological testing of antirachitic activity.

Immediately after irradiation an aliquot part of the fluid to be tested was dissolved in 5 cc. of olive oil and the alcohol or ether removed *in vacuo* at room temperature. This solution was then suitably diluted with olive oil so that the required amount of irradiated ergosterol was contained in 20 mm.^{3} of oil.

The test animals were young albino rats of about 40 g. weight and the diet used was the modified diet 84 [Rosenheim and Webster, 1926]. In most cases the protective method was used, the animals being kept on the diet for 21 days, during which period daily doses of the test solutions were given to all except the control animals. At the expiration of this time the animals were killed and the left hind leg was dissected out and photographed with a soft X-ray tube. The limbs from each litter of rats were photographed on one plate, and the degree of rickets in each rat was carefully estimated.

Although litter mates react fairly consistently to rachitic diets and antirachitic treatment, different litters show much less consistency. To overcome this variation each litter of rats was divided into two groups; one group received graded doses of the solution under test and the other graded doses of a standard solution. Comparison of corresponding members of the two groups then gave a measure of the activity of the solution under test in terms of the standard.

Each rat received one dose per day, Sundays excepted, administered with Wright's capillary pipette. The use of a standard preparation for the assay of vitamins has also been described by Coward [1928].

The standard solution of irradiated ergosterol was prepared as follows. A 0·1 % solution of ergosterol in dry ether was exposed to the radiations of a K.B.B. silica mercury vapour lamp taking 2·5 amp. at 125 volts for 40 minutes. The ether solution was contained in the standard silica cell 1 cm. thick at a distance from the lamp of 8 inches. After exposure the ether was rapidly removed on the steam-bath in a current of nitrogen and the residue dissolved in olive oil, the concentration being 0·01 %. This solution was kept frozen solid at about 0° and dilutions were made when required. Fresh dilutions were made for every set of experiments, so that no solution was kept at room temperature longer than 3 weeks.

Results of antirachitic tests.

All products appeared of approximately equal activity except those obtained in liquid oxygen or nitrogen, which were markedly less active. The results obtained from + 78° to − 18° are based on tests on about 170 rats, and are probably fairly reliable for this class of work. A difference of 100 % between any two fluids would probably have been evident, and a regular difference of this magnitude between filtered and unfiltered light would be most unlikely to have escaped detection.

The products obtained at − 183° and − 195° are definitely less active. In a series of trials products have been obtained varying from one-tenth to one-half of the standard strength, but in some series both the antirachitic activity and the spectrographic changes in the solutions are less than in others. The cause of this variability is not yet certain, but may be turbidity of the liquid nitrogen or oxygen, caused by particles of ice with which it is liable to become contaminated.

A further test of the filtered light at 30° was made by prolonged irradiation of 0·1 % solutions of ergosterol in ether for periods of 1, 4, and 63 hours respectively.

The first two fluids showed a maximum standard activity, while that given 63 hours' exposure (equivalent to about 1½ hours' exposure of 0·01 % solution to unfiltered light) was less than one-quarter as active.

It seems reasonable to conclude that the exclusion of wave-lengths shorter than 265$\mu\mu$ does not seriously alter the ratio of rates of production and destruction of vitamin D. This suggests, but does not prove, that vitamin D either has strong absorption for wave-lengths longer than 265$\mu\mu$, or has not great absorption for wave-lengths of 230 to 250$\mu\mu$ as suggested by previous workers.

The lack of marked effect of changes of temperature between + 78° and − 18° on the equilibrium, shows that the temperature coefficients of the changes causing production and destruction are not widely different, and the very moderate effect of lowering the temperature to − 180° suggests that the temperature coefficient of both reactions is very small, and hence that both reactions are directly photochemical in nature.

II. *Some properties of the products of irradiation after removal of unchanged ergosterol.*

The products remaining after the removal of unchanged ergosterol from solutions irradiated for short periods only are being investigated. By this means it was hoped to secure potent preparations of vitamin D free from ergosterol and containing a reduced proportion of inactive decomposition products. The technique was as follows. A 0·1 % or 0·15 % solution of ergosterol in alcohol was irradiated for periods of ½ minute to 20 minutes in a layer 10 mm. thick at 3 to 6 inches from a mercury vapour lamp. The irradiated solution was treated (either with or without previous concentration by evaporation

in vacuo) with a 1 % solution of digitonin in 95 % alcohol, using an amount of digitonin equal to 5 times the weight of ergosterol present before irradiation, *i.e.* an excess of more than 25 % over the quantity theoretically needed for precipitation. Enough water was added to bring the total concentration of water up to 10 %. The mixture was allowed to stand overnight and then filtered. The precipitate of ergosterol digitonide was rejected. The filtrate was in some cases used for spectrographic and biological tests, but always contained small traces of ergosterol. To remove these the solution was evaporated to dryness *in vacuo* at about 40° (thus ensuring the complete precipitation of ergosterol during concentration), and the residue was then extracted with dry ether, in which digitonin and ergosterol digitonide are practically insoluble. The solution was used as such, or evaporated to dryness in order to weigh the residue. All these operations were carried out as far as possible in an atmosphere of nitrogen and at temperatures never exceeding 50°. The application of this process to non-irradiated ergosterol yields only traces of oily substances which are without appreciable absorption.

The products so obtained formed a transparent glassy hard solid of indefinite M.P. beginning about 30°, at times colourless, but more often contaminated with a yellow pigment which appeared during the original solution in alcohol of certain samples of ergosterol [Tanret, 1908]. The product is soluble in approximately its own weight of alcohol at 30°. This is in striking contrast to the small (0·2 %) solubility of ergosterol in alcohol, and is a proof of the efficiency of the process described above for removing ergosterol. The product is also very soluble in ether, chloroform and light petroleum, but insoluble in water. It is moderately soluble in methyl alcohol containing 10 % of water, and this mixture was used in attempts to fractionate the mass. It shows high antirachitic activity. This appears to vary in different samples prepared on different occasions. The highest observed activity appears to have been about twice that of the standard described above. The absorption spectrum varied considerably in different samples, and is discussed in the next section.

III. *Absorption spectra of products of irradiation.*

Preliminary studies of absorption have been made by a photographic method, using a modification of the technique described by Dobson, Griffith and Harrison [1926]. The results obtained were in general concordant to about 5 %, but difficulty was found in controlling the voltage on the lamp used, and at times a larger error occurred. Greater sources of error were found in the presence of traces of the yellow pigment referred to above, which showed strong absorption between 300 and 400$\mu\mu$. For these reasons the curves shown are only rough approximations, and no attempt has been made to locate exactly the heads of the individual bands.

Curve 1 (Fig. 7) and (Plate III, fig. 1) show the absorption of the ergosterol used.

Curve 2 (Fig. 7) and (Plate III, fig. 2) show the absorption of a practically colourless sample of the product obtained after the removal of ergosterol as described above.

Curve 3 (Fig. 7) shows the absorption of another sample of the product.

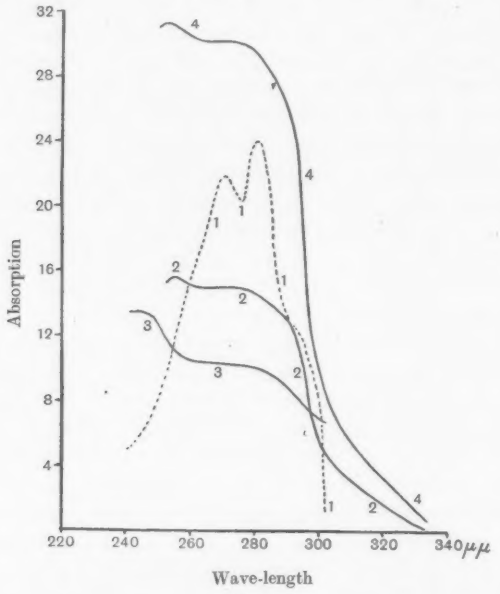

Fig. 7.

These products show strong absorption for wave-lengths shorter than 290$\mu\mu$ and in these samples show no maximum between 265 and 280$\mu\mu$ in contrast to ergosterol and to certain other samples of products in which a maximum occurred between 300 and 330$\mu\mu$. The absorption is, however, much greater than that of ergosterol. The above features can be seen by inspection of Plate III, figs. 1 and 2, and are of much interest in conjunction with the following phenomena, which can be observed during the earliest stages of the irradiation of ergosterol.

If a solution of ergosterol be examined after short periods of irradiation, an increase in absorption is noteworthy, in contrast to the decrease recorded by many workers after longer periods of radiation. In a 10 mm. layer of 0·2 % solution this increase is very striking at 313$\mu\mu$ (Plate IV, fig. 3), while in 0·1 % solution it is obvious at 302$\mu\mu$ (Plate IV, fig. 4), and in 0·01 % solution it is evident at 290 and 265$\mu\mu$ and visible at 253$\mu\mu$ (Plate IV, fig. 5) after 4 minutes' irradiation. In 0·05 % solution it is just visible in the intervening wave-lengths between 290 and 265$\mu\mu$ after about 2 minutes' irradiation. The increase is at its maximum after a period depending on the concentration

of solution as well as on the intensity of radiation. Under our conditions this maximum occurs after about 6 minutes in 0·01 % solution, or an hour in 0·1 % solution (the exact times have not been measured). On further irradiation the absorption decreases steadily to the almost complete disappearance often recorded. In fig. 5 (Plate IV) a marked decrease in absorption is seen after 20 minutes' irradiation.

These changes do not occur if a filter of thick window-glass is interposed between the lamp and solution, but do occur if filters of chlorine or alcoholic cobalt chloride are used, although the latter modifies the changes. They appear unaffected by bubbling either oxygen or nitrogen through the solution during irradiation, or by the use of ether as solvent instead of alcohol. The most simple explanation of them is the hypothesis that ultra-violet irradiation has as an initial (or early) effect the formation from ergosterol of products showing strong absorption between 313 and 250$\mu\mu$. The intensity of this absorption markedly exceeds that of ergosterol between 320 and 290$\mu\mu$, and between 265 and 250$\mu\mu$, and slightly exceeds it between 290 and 270$\mu\mu$. (We have only worked with a mercury vapour spectrum and so have not excluded the possibility of narrow troughs between bands, in which the absorption may be less than that of ergosterol.) This hypothesis is strongly supported by the absorption of the products described above which remain after the removal of ergosterol from an irradiated solution. For, although these products show an absorption which only exceeds that of ergosterol between 300 and 330$\mu\mu$, if it is assumed that the products are a pure absorbing substance, it is much more probable that the products are a mixture of absorbing and non-absorbing substances. If such non-absorbing substances form 40 % of the sample whose absorption is given in curvé 2 (Fig. 7), the true absorption curve of the absorbing product would be that shown in curve 4 (Fig. 7). Such an absorption would satisfactorily account for the increase in absorption shown in the first few minutes of the irradiation of ergosterol.

IV. *Possible absorption spectrum of vitamin D.*

We have made numerous attempts to find a quantitative relation between the magnitude of the absorption coefficients of the products described above and their antirachitic activity. Owing to the difficulties of such tests, no exact relationship has been proved or disproved, but the following conclusions can be stated.

1. If a 0·1 % solution of ergosterol in alcohol or ether is irradiated at room temperature under the conditions described in Section I, products having an antirachitic activity and absorption of the type described are present to a small extent after 30 seconds' irradiation, and to a marked extent after 1 minute's irradiation.

2. After 10 minutes' irradiation both properties are increased to something like 10 times the former values (Plate IV, fig. 6).

3. Irradiation for periods of between 10 and 60 minutes increases both properties somewhat further, to about 2 or 4 times their value after 10 minutes' irradiation.

4. Thus there is evidence that both antirachitic activity and absorption are produced at approximately the same rate, and that both begin in the earliest stages.

5. If the products of irradiation after removal of ergosterol are exposed to ultra-violet light, both antirachitic activity and absorption decrease, and both disappear almost entirely after between 3 and 5 hours' irradiation under the above conditions.

6. We have not yet succeeded in obtaining a product showing either antirachitic activity without the type of absorption described, or *vice versa*. Each property has always been accompanied by the other. There is thus some reason for thinking that absorption of the type shown in curve 4 (Fig. 7) may be a property of vitamin D. This conclusion is, however, only one of several possible explanations of the observed phenomena, and is not easy to reconcile with the observations of Morton *et al.* or of Pohl [1926]. A more probable explanation, which we hope to test shortly, is that the irradiation of ergosterol produces two substances in succession, of which the first has an absorption maximum at about 280 or 290$\mu\mu$, and the second a maximum at about 230$\mu\mu$, and that the former is vitamin D. This hypothesis would explain the variable absorption of the products obtained after removing ergosterol from irradiated mixtures, and would also fit the absorption changes found by us in the early stages of the irradiation of ergosterol and the changes found by the other authors quoted in the later stages. On any hypothesis the products of irradiation must show considerable antirachitic activity until some time after the practical disappearance of ergosterol, and this makes the evidence attributing absorption at 240$\mu\mu$ to vitamin D open to question unless a quantitative relation between such absorption and antirachitic activity can be shown. As arguments suggesting a connection between vitamin D and absorption at 280$\mu\mu$ may be quoted the very early production of marked antirachitic activity on irradiation of ergosterol, and the fact that in our experiments we have always found marked absorption at 290 and 300$\mu\mu$ in products showing antirachitic activity.

We desire to express our thanks to Dr G. M. Dobson, F.R.S., for advice on photometry and for the use of his own photometer.

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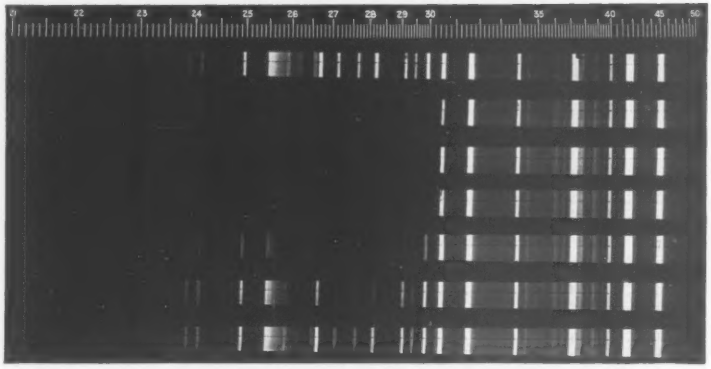

Fig. 1. Ergosterol in alcohol.

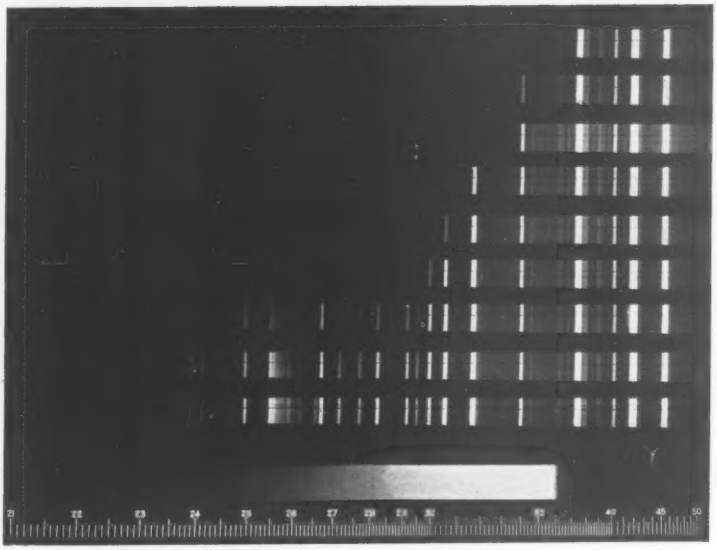

Fig. 2. Products of irradiation after removal of ergosterol.

In Figs. 1 and 2 it should be noted (*a*) that in Fig. 1 (ergosterol) absorption is still marked at 270 to 290 in a concentration of 0·0062 % although line 254 is clearly visible, whereas in Fig. 2 at concentration 0·0084 % the absorption between 270 and 290 is no greater than that at 254: (*b*) that in Fig. 2 at concentration 0·067 % the line 302 has entirely disappeared, although in Fig. 1 this line is still prominent even at 0·1 % concentration.

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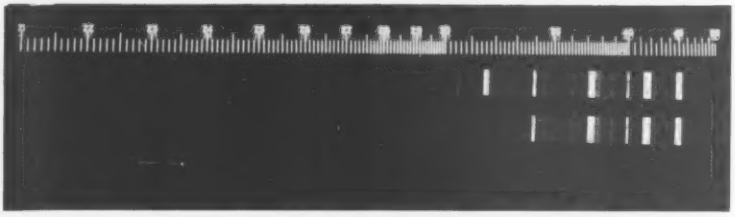
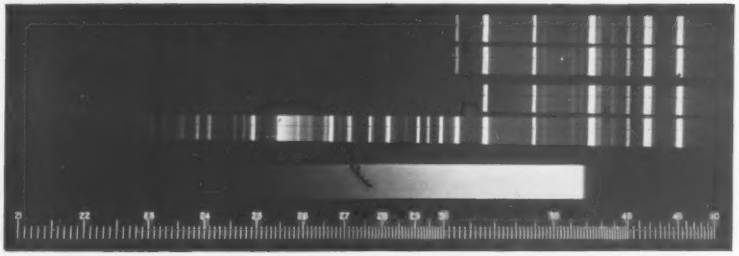


Before irradiation

After „

Fig. 3. Ergosterol 0·2 % in ether.



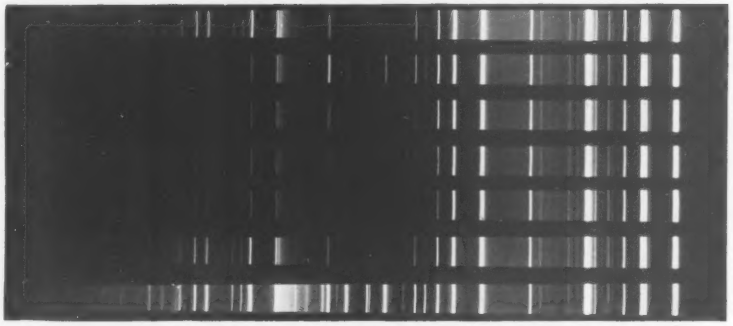
Before irradiation

After 1 min. irradiation

„ 10 „ „

Ether

Fig. 4. Ergosterol 0·1 % in ether.



Before irradiation

After 20 min. irradiation

„ 13 „ „

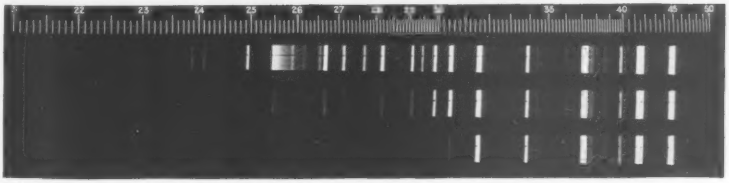
„ 8 „ „

„ 4 „ „

Before irradiation

Spectrum of lamp only

Fig. 5. Ergosterol 0·01 % in alcohol.



Ether

Products of 1 min. irradiation

Products of 10 min. irradiation

Fig. 6. Products of irradiation after removal of ergosterol

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CLIII. THE DESTRUCTIVE ACTION OF HEAT ON INSULIN SOLUTIONS.

By AUGUST KROGH and AXEL MARIUS HEMMINGSEN.

From the Laboratory of Zoophysiology and the Nordisk Insulin Laboratory, Copenhagen.

(*Received August 26th, 1928.*)

We have endeavoured to determine the relationship between temperature and destruction of sterile watery buffered solutions of insulin (about 15 international units per mg.) and to find a mathematical expression for it. According to the law of mass action we might expect that the rate at which insulin is destroyed at a constant temperature at any moment should be proportional to its concentration as expressed by the well-known equation

$$k = \frac{1}{t} . \log \frac{a}{a-x}$$

where k is the velocity constant, a the initial concentration, and x the amount destroyed at the time t. This expression can be used to evaluate k, the quantity $(a - x)$ left at a given time t being estimated by biological standardisation.

Experiments were carried out at 50°, 60°, 80°, 100° and 117°.

The constant temperatures were obtained by using an electrical incubator (at 50°, 60° and 80°), boiling water or vapour from boiling water (100°) and vapour from boiling normal primary butyl alcohol (117°).

For the experiments at 50°, 60°, 80° and 100° the solution was placed in rubber-capped vials, whereas at 117° we used test-tubes (Jena Fiolax glass) sealed in the blow-pipe. They were placed at the constant temperatures and after the lapse of a certain period of time they were taken out and instantly cooled in water. The potency of each sample was compared with the potency of a sample of the same solution which had been left in the ice-chest, where no measurable destruction takes place within very long periods of time. The constants evaluated from the experiments are given in Table I.

In order to decide whether or not the equation employed holds good for the destruction process we have made experiments at 60° (one experiment), 100° (three experiments), and 117° (two experiments) in which samples (each in a separate vial) were taken out at various periods of time. By placing a thermometer in one of the vials used at 100° and a metallic alloy melting just below 117° in one of the tubes used at that temperature it was found that the time elapsing before the liquid in the tubes became heated to the surrounding temperature could have no appreciable influence on the result (*i.e.* the error

of the time of heating would not exceed 1 % even in the shortest experiment). The results of the experiments are given in Table I.

Table I.

Temp. ° C.	t (hours)	Initial p_H*	$\frac{a-x}{a}$	k	a (units per cc.)	Brand†	Test†	No. in Fig. 2
50·1	623·0	4·5	0·66	0·000296	20	Leo	r.	1
50·1	623·0	3·5	0·60	0·000360	15	Leo	r.	2
60·0	192·0	4·0	0·52	0·00147	20	Leo	m.	3
60·0	360·0	4·0	0·39	0·00113	20	Leo	m.	4
60·0	696·0	4·0	0·116	0·00134	20	Leo	m.	—
60·0	1030·0	4·0	0·076	0·00109	20	Leo	m.	—
60·0	143·0	4·5	0·77	0·000797	—	Leo	r.	5
60·3	181·0	3·5	0·59	0·00128	—	Leo	r.	6
60·0	308·5	—	0·47	0·00107	—	Leo	r.	7
80·0	16·0	—	0·82	0·00537	—	B.D.H.	r.	—
79·7	16·2	3·5	0·52	0·0178	—	Leo	r.	8
81·0	20·0	—	0·36	0·0219	—	Leo	r.	9
100·0	1·0	4·0	0·74	0·131	20	Leo	m.	—
100·0	2·0	4·0	0·43	0·183	20	Leo	m.	—
100·0	3·08	4·0	0·425	0·121	20	Leo	m.	—
100·0	4·0	4·0	0·29	0·134	20	Leo	m.	—
100·0	6·0	4·0	0·35	0·076	20	Leo	m.	—
100·0	8·0	4·0	0·38	0·053	20	Leo	m.	—
100·0	2·5	4·0	0·475	0·129	20	Leo	m.	10
100·0	8·0	4·0	0·195	0·089	20	Leo	m.	—
100·0	1·73	4·0	0·50	0·174	40	Leo	m.	11
100·0	3·5	4·0	0·27	0·163	40	Leo	m.	—
100·0	5·26	4·0	0·228	0·122	40	Leo	m.	—
100·0	7·0	4·0	0·125	0·129	40	Leo	m.	—
100·0	9·3	4·0	0·074	0·121	40	Leo	m.	—
100·0	10·53	4·0	0·046	0·127	40	Leo	m.	—
100·0	2·0	—	0·50	0·150	—	B.D.H.	r.	12
100·0	2·0	4·0	0·57	0·124	—	Leo	r.	13
100·0	2·0	3·5	0·60	0·111	—	B.W.	r.	14
117·0	0·542	4·0	0·415	0·705	20	Leo	m.	15
117·0	1·04	4·0	0·355	0·432	20	Leo	m.	—
117·0	2·21	4·0	0·177	0·34	20	Leo	m.	—
117·0	2·73	4·0	0·135	0·319	20	Leo	m.	—
117·5	1·0	4·0	0·332	0·478	40	Leo	m.	16
117·5	2·05	4·0	0·18	0·362	40	Leo	m.	—
117·5	3·0	4·0	0·10	0·33	40	Leo	m.	—
117·5	4·0	4·0	0·048	0·33	40	Leo	m.	—
117·5	5·0	4·0	0·029	0·308	40	Leo	m.	—

* At the time when the experiments were made the colorimetric method of p_H determination was used (bromophenol blue). When later on the hydrogen electrode was employed it turned out that a considerable correction had to be made. Thus in reality the values of $p_H = 4$ in the table correspond to $p_H = 3{\cdot}2$ as measured by the hydrogen electrode.

† Abbreviations: B.D.H., British Drug Houses; B.W., Burroughs Wellcome; r., rabbit test; m., mouse test.

From the equation quoted above the logarithm of undestroyed insulin $(a - x)$ can be expressed as a linear function of heating time, t,

$$\log (a - x) = - kt + \log a.$$

Since a is the initial concentration, $\log a$ is constant, and accordingly the results obtained from the experiments at 60°, 100° and 117° should be represented by a straight line when plotting heating time against the logarithm of undestroyed insulin. In Fig. 1 this is illustrated in the case of one of the experiments (at 117·5°). Apparently a line connecting the points would in

some of the experiments have a tendency to bend upwards during its course, indicating that the destruction becomes relatively slower as the activity decreases, but in no cases except perhaps in one experiment at 100° can this be said with certainty. The velocity constant calculated from different points at the same temperature shows variations of 50–100 % (see Table I) probably for the most part owing to experimental errors, but perhaps also to some extent because the equation fails to express quite exactly the mechanism of the process.

However, the variations of the constant calculated for the same temperature are negligible when compared with the difference between the averages of the constants calculated for different temperatures, *e.g.* the constant at

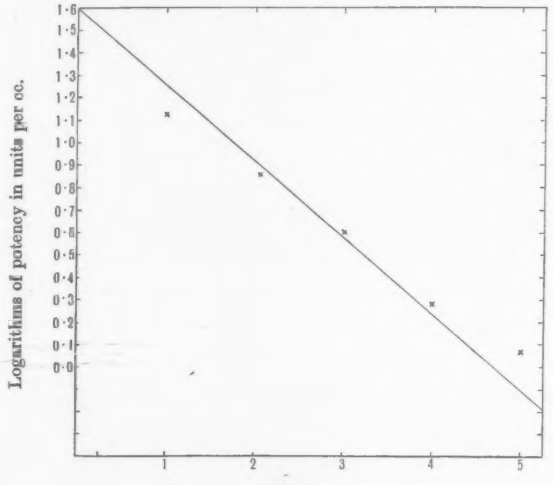

Fig. 1.

100° is a hundred times larger than the constant at 60°, and the constant at 117° is about three times as large as the constant at 100°.

In spite of the rather large dispersion of the constants for each temperature the wide range of temperatures employed appears sufficient as regards the determination of the exact relation of velocity constant to temperature.

Arrhenius's relation between absolute temperature T and the velocity coefficient k can be written in terms of a linear relation

$$\log_{10} k = -\frac{0{\cdot}43.\mu}{2}.\frac{1}{T} + \text{constant},$$

and its applicability can be tested by plotting log k against $1/T$.

As seen in Fig. 2 the process follows closely the law of Arrhenius, at least within the limits of temperatures employed. Only constants evaluated from

nearly the same stage of destruction, namely half destruction, have been employed since this seems to be the most proper way of comparison.

In view of the wide divergence of temperatures examined it seems fairly probable that the relation found will be valid over an even wider range of temperatures. Assuming this, the time taken for the activity to fall 5, 10 and 50 % has been calculated for a series of lower temperatures, at which it is difficult to make experiments owing to the slow rate of destruction. The results are given in Table II.

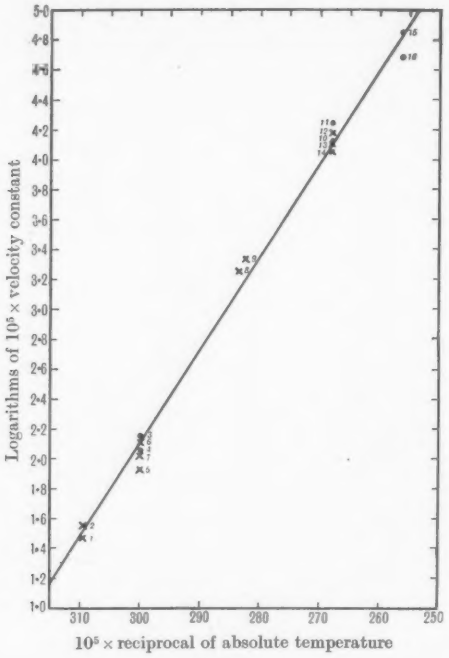

Fig. 2.

Table II.

	Time taken for the activity to fall		
	5 %	10 %	50 %
40°	¼ month	1 month	½ year
30°	2 months	4 months	2 years
20°	9 „	1½ years	10 „
10°	4½ years	8½ years	56 „
0°	29 „	58 „	377 „

Since the values given in Table II are extrapolated values, the value for 0° will be much less accurate than those for 30° and 40°. Judging from calculations

based upon displacement of the straight line in Fig. 2 into extreme and improbable situations the accuracy of the values for 0° is 50–100 %, and for 30° about 25 %.

The value of the constant μ calculated from the straight line drawn in Fig. 2 is 28,300, which is a very high value as compared to the values met with in most chemical reactions. Generally μ is between 10,000 and 17,000. High values of μ are by some authors especially ascribed to monomolecular reactions. Whether the apparent change in the relative velocity during a single destruction experiment may be due to the formation of products inhibiting the destruction or to some other reason, must be the aim of future research. We ought to mention that there is invariably a precipitation of inactive material at this p_H during the heating process and that there is a shift of p_H during the destruction process in spite of the buffer. Applying the hydrogen electrode at 18° to a solution before and after 2 hours' heating at 100° we found a shift of p_H from 3·24 to 3·40. As standard electrode the Veibel quinhydrone electrode was used [Biilmann, 1925].

The influence of temperature on the buffer action of the solutions employed was tried by placing the same solution at 18° and at 70° (in a water-bath covered with oil) and applying a hydrogen electrode. As a standard electrode at 70° another hydrogen electrode was used in 0·01 *N* HCl + 0·09 *N* KCl which was supposed to have the same p_H at 18° and 70°. Owing to the high vapour pressure at 70° the experiment could not be of long duration. A constant potential was rapidly obtained corresponding to $p_H = 3{\cdot}31$. The same solution showed $p_H = 3{\cdot}24$ at 18°. Applying to another solution the hydrogen electrode at 18°, 25° and 37° (in water thermostats), using the Veibel quinhydrone electrode as a standard electrode and applying its temperature coefficient as quoted by Biilmann [1925], the shift of p_H was found to be 0·01. We conclude from these experiments that there is no large shift of p_H caused by temperature or taking place in the course of a destruction experiment. But as we know nothing exactly about the influence of p_H on the rate of destruction the shifts of p_H might to some extent be the cause of the dispersion of the constants.

For the purpose of determining the influence of shifts of p_H on the destruction rate a number of solutions were colorimetrically adjusted to different values of p_H and supplied with buffers, and the p_H values were checked by hydrogen electrode measurements. The solutions adjusted to p_H about 4 and 6·5 have in most cases been slightly opalescent.

The buffers added were such as have no temperature coefficient according to Walbum [1920], at least between 18° and 70°, namely the citrate-HCl mixtures and the phosphate mixtures of Sørensen [1909]. The influence of temperature on the buffer action of insulin itself is very slight according to the experiments quoted above (when the buffer added was also one that had no temperature coefficient between 18° and 70°).

As seen in Table III the colorimetric method and the electrometric method do not agree when bromophenol blue and bromocresol green are used as

indicators. All the electrometrically obtained values given are calculated from potentials which had attained a constant value (*i.e.* constant within about half a millivolt or less in the course of a quarter of an hour). For the calculation of the values of p_H 1–3 the Bjerrum [1911] extrapolation formula has been applied in order to eliminate the liquid junction potential, first using 3·5 *N* KCl and then using 1·75 *N* KCl to connect the two electrodes. In the other cases only 3·5 *N* KCl was used. For all the measurements one of two platinum electrodes was used which had been shown to produce the same potential when used in the same solution.

Table III.

Batch	p_H Color.		p_H Electr.	Buffer (*i*)	*t* (hours)	*a*	*a* − *x*	*k*	Temp. °C.
A	1·2	(*a*)	1·10	1·0	0·75	18·7	11·9	0·261 (*g*)	100·8
B	1·2	(*a*)	1·17	1·0	1·0	20·0	11·0	0·26 (*g*)	100·2
B	1·2	(*a*)	1·17	1·0	1·225	20·0	9·9	0·249 (*h*)	100·6
C	2·0 ±0·1	(*a*)	1·73	0·25	1·975	20·0	11·1	0·130 (*h*)	100·1
D	2·0 ±0·1	(*a*)	1·79	0·25	1·975	20·0	11·5	0·122 (*h*)	100·1
E	1·9	(*a*)	1·78	0·25	4·0	20·0	5·1	0·149 (*g*)	100·4
A	1·9	(*a*)	1·76	0·50	2·0	13·3	8·1	0·108 (*g*)	100·0
C	2·45 ±0·15	(*a*)	2·42	0·25	1·975	20·0	10·9	0·134 (*h*)	100·1
D	2·45 ±0·15	(*a*)	2·34	0·25	1·975	20·0	11·8	0·116 (*h*)	100·1
E	2·8 ±0·5	(*a*), (*b*)	—	0·25	3·987	20·0	8·6	0·092 (*g*)	100·4
A	3·0	(*a*), (*b*)	2·88	0·57	2·0	11·0	6·1	0·128 (*g*)	100·0
C	4·2 ±0·2	(*c*)	3·63	0·25	1·975	20·0	10·8	0·135 (*h*)	100·1
D	4·2 ±0·2	(*c*)	3·65	0·25	1·975	20·0	9·8	0·157 (*h*)	100·1
E	4·0	(*d*)	3·58	0·25	3·975	20·0	7·2	0·112 (*g*)	100·4
C	6·5 ±0·2	(*d*)	6·75 (*f*)	0·25	0·975	20·0	7·4	0·432 (*h*)	100·6
C	6·5 ±0·2	(*d*)	6·75 (*f*)	0·025	0·975	20·0	10·3	0·289 (*h*)	100·6
B	7·45 ±0·3	(*e*)	7·43	0·80	0·0833	20·0	12·7	2·36 (*h*)	100·2

(*a*) thymol blue; (*b*) bromophenol blue; (*c*) bromocresol green; (*d*) bromophenol blue; (*e*) cresol red; (*f*) in reality this solution was not electrometrically checked: three other different solutions prepared in a similar way were colorimetrically adjusted to $p_H = 6{\cdot}5 \pm 0{\cdot}2$ and showed $p_H = 6{\cdot}81$, 6·75 and 6·70 with the hydrogen electrode; (*g*) sealed test-tubes; (*h*) rubber-capped vials; (*i*) buffer content expressed in proportion to buffer content of Sørensen's standards of the same p_H.

The solutions were submitted to a temperature of 100° for a period of time supposed to cause half destruction and then compared with a sample of the same solution which had been submitted to 100° for 1·5 minutes. Since this is the time taken for the vials to reach 100° the error of a too low constant in short experiments will be avoided provided that 1·5 minutes is subtracted from the apparent heating time when evaluating the constant.

The results of these experiments are given in Table III and Fig. 3. It is seen from the figure that the influence of the slight p_H variations in the destruction experiments is too small to account for the dispersion of the constants in either Fig. 2 or 3, when p_H is about 3. For the purpose of comparing the dispersion of the constants in Figs. 2 and 3 the logarithms of the constants have been chosen as ordinates also in Fig. 3, and the results from the experiments at 100° in Fig. 2 have been inserted at p_H 3·2 in Fig. 3.

For the purpose of mutual control some of the estimations of insulin activity have been performed by the rabbit blood-sugar test as described by Marks [1926] and others by the mouse convulsion test as described by Krogh

and Hemmingsen [1926]. In Fig. 2 the points obtained from the mouse method (marked ●) show good agreement with the points obtained by the rabbit method (marked ×).

A closer study reveals that the dispersion of the constants for each temperature is larger than might be expected from the accuracy usually obtained with the methods of assay under consideration. This must be ascribed to unknown sources of error in the destruction experiments. It ought also to be mentioned that single determinations of partly destroyed samples show a tendency to larger dispersion than do undestroyed samples, perhaps because of different conditions of resorption in the animals.

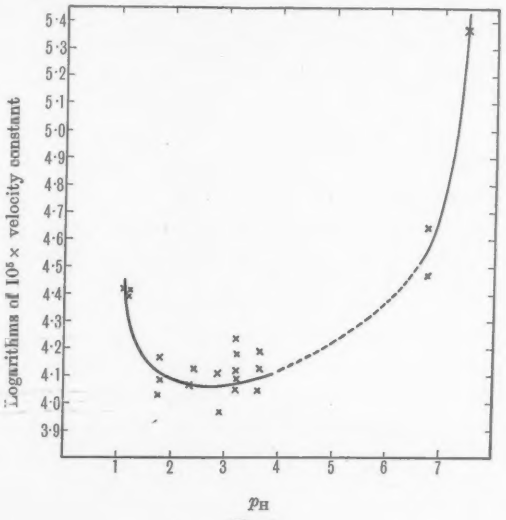

Fig. 3.

SUMMARY.

1. The destruction of insulin at a constant temperature is found to follow fairly well the law of monomolecular reactions, the rate being at any moment proportional to the concentration.

2. The relation of rate of destruction to temperature can be well expressed by the formula of Arrhenius. The critical thermal increment amounts to 28,300.

3. The rate of destruction is not appreciably altered by shifts of p_H when this is between 2 and 4.

We are greatly indebted to Prof. E. Biilmann and Mr A. Klit for the use of the apparatus for the electrometric determination of p_H at the Chemical Laboratory of the University of Copenhagen, and for the benefit of their special knowledge in this field of work.

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CLIV. ON THE MEASUREMENT OF CHLORINE ION POTENTIALS IN THE PRESENCE OF PROTEINS.

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From the Physiology Laboratory, Oxford.

(*Received August 23rd, 1928.*)

Calomel and silver-silver chloride electrodes have been used extensively in the physical chemistry of the proteins for determining the concentration of chlorine ions in the presence of proteins [Bugarsky and Liebermann, 1898; Manabe and Matula, 1913; Oryng and Pauli, 1915; Pauli and Matula, 1917; Loeb, 1922; Northrop and Kunitz, 1924]. A few attempts have also been made to use these electrodes for chlorine determinations in biological fluids. Barkus [1924] reported some estimations of Cl′ in gastric juice using Ag-AgCl electrodes; Neuhausen and Marshall [1922] used similar electrodes to find out whether all the chlorine in serum is free and diffusible; and Brown and Hill [1921] attempted to show the difference in chlorine concentration between oxygenated and reduced blood by the same means. On the other hand Warburg [1922] stated that such electrodes (concentration chains of the second order) in the presence of protein will not give potentials that conform with the Nernst equation, and that their use is therefore unsound; he argued this on purely theoretical grounds and brought no experimental evidence in support of it. In the following experiments an attempt was made to use the silver-silver chloride electrode for the determination of chlorine in human blood-plasma; it proved unsuccessful but, especially in view of Warburg's criticism, the observations seem worth recording.

Apparatus and technique.

The following chain was used:

Ag-AgCl | 0·02 molar KCl | "unknown" | AgCl-Ag.

The electrodes were made of pure silver wire, bent twice at their lower ends and covered over the bent parts with an electrolytically deposited layer of silver chloride; they were mounted on rubber corks fitted into glass electrode vessels with side tubes for making electrical contact with each other. In one vessel were placed about 4 cc. of a standard solution of pure 0·02 *M* KCl, in the other a similar quantity of the plasma or other solution under investigation; a direct liquid junction was effected by bringing the free ends of the side tubes

of the vessels into apposition, and slipping a short piece of rubber tubing over them. The potential difference between the two electrodes was then measured with a Cambridge and Paul direct-reading potentiometer, using a recently checked Weston cell; the result could be read to 0·0001 volt, which gave a deflection of 1 mm. on the galvanometer scale.

Before every experiment six electrodes were prepared by being cleaned and re-coated with fresh layers of silver and silver chloride. Individual variations between electrodes were then avoided by choosing two which gave no P.D. when immersed in the same KCl solution. These two were set up in the electrode vessels, which along with the standard cell were kept at a uniform temperature of $20° \pm 0·02°$, in an air thermostat of the kind described by Clark [1922]. Potentiometer readings were taken at varying intervals of time after the closure of the liquid junction.

Observations on pure KCl solutions.

A preliminary series of observations was made on pure KCl solutions of known concentration, as a test of the reliability and accuracy of the apparatus and technique. The solutions were all approximately 0·1 *M*. The potentiometer readings sometimes showed fluctuations of a few tenths of a millivolt in the first 10 minutes after closure of the liquid junction, but after that in every case they remained steady to the nearest 0·0001 volt. They were observed at frequent intervals for periods varying from 25 to 140 minutes; breaking the junction and making a fresh one did not cause any change in the reading.

The logarithms of the concentrations of the solutions, and the E.M.F. obtained are given in Table I, and plotted in Fig. 1. The points lie on a straight line, as might be expected (over this narrow range of concentrations) from the equation for the E.M.F. of a concentration chain of this kind, viz.

$$E = \frac{2\eta RT}{nF} \ln \frac{c_1 \cdot \gamma_1}{c_2 \cdot \gamma_2},$$

where E = E.M.F. in volts; η = transference number of cation = 0·496 for K; R = gas constant = 8·316; T = absolute temperature; n = valency; F = Faraday's constant; c_1, c_2 = concentrations of anion; γ_1, γ_2 = activity coefficients of anion.

From this straight line the E.M.F. corresponding to a Cl′ concentration of 0·1 mol. per 1000 g. water can be read. It is 0·0370 volt. If this be substituted for E in the above equation, then since the ratio c_1/c_2 is known to be 0·10/0·02 and the values of the constants are known, γ_1/γ_2 can be calculated and is found to be 0·876. This agrees with the results of Noyes and McInnes [1920] who obtained the values $\gamma_1 = 0·745$ and $\gamma_2 = 0·849$ (by interpolation), giving a ratio of 0·878.

The preliminary series of observations therefore showed that the apparatus and technique were adequate; the E.M.F.'s obtained with known KCl solutions were steady, reproducible (as shown by the straight line in Fig. 1) and in agreement with standard observations.

Observations on KCl solutions containing protein.

The next step was to find out whether steady and reproducible E.M.F. could be obtained from solutions of known chlorine concentration, containing protein. For this purpose sheep or ox serum was dialysed in collodion tubes in a cool room against either 0·03 *M* sodium bicarbonate, or 0·03 *M* buffer solution of sodium acetate and acetic acid of p_H 5·5, until the dialysate gave no precipitate with silver nitrate; in one or two cases the protein solution was also tested for chloride by ashing and extraction with dilute nitric acid and found to be chlorine-free. The final concentration of the protein solution was determined by micro-Kjeldahl estimation of the nitrogen, and known amounts of pure KCl were then added by weight. Four control solutions were also prepared by dissolving KCl in the bicarbonate or acetate solutions used in the dialysis.

Observations of E.M.F. were made on 20 solutions in all; their composition and the final readings obtained are shown in Table I (Nos. 11 to 30).

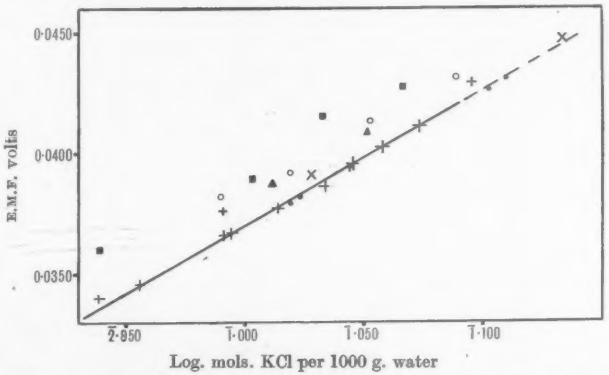

Fig. 1. The relation of the E.M.F. to the logarithm of the concentration of KCl (Table I).

+ : Solutions 1 to 9, containing nothing but KCl in water.
● : Solutions 11 to 14, containing acetate or bicarbonate
▲ : Solutions 15 to 17, containing bicarbonate and 2·7 % protein.
■ : Solutions 27 to 30, containing bicarbonate and 8·5 % protein.
+ : Solutions 20 to 22, containing acetate and 3 % protein.
× : Solutions 18, 19, containing acetate and 7·8 % protein.
○ : Solutions 23 to 26, containing acetate and 10 % protein.

The E.M.F. of the solutions containing salts but no protein, Nos. 11 to 14, usually fell a few tenths of a millivolt in the first 10 or 15 minutes after closure of the liquid junction, and thereafter remained satisfactorily steady.

In a solution containing protein, however, the E.M.F. was by no means steady. It was found that a slight mechanical disturbance, such as tapping the upper end of the electrode dipping into the protein solution, would regularly cause a sharp rise in the E.M.F.—a millivolt or so—followed by a gradual fall.

This difficulty was overcome by keeping the electrode in a state of continual vibration. The gong of an electric bell was removed and its clapper replaced by a flagellum of iron wire about 8 inches long. The brass connecting piece carried by the top end of the electrode wire was covered with insulating tape, and the end of the flagellum brought up against it. When the bell current was turned on, the "whipping" of the brass piece by the flagellum kept the upper and the lower ends of the electrode wire in a state of continuous agitation, which had the additional advantage of stirring the liquid in the electrode vessel.

Table I.

No.	Concentration of protein %	Salts present (other than KCl) 0·03 *M*	Log. mols. KCl per 100 g. water	E.M.F. volts
1	—	—	$\bar{2}$·9943	0·0367
2	—	—	$\bar{2}$·9381	0·0340
3	—	—	$\bar{1}$·0585	0·0402
4	—	—	$\bar{2}$·9912	0·0366
5	—	—	$\bar{2}$·9558	0·0346
6	—	—	$\bar{1}$·0134	0·0377
7	—	—	$\bar{1}$·0339	0·0386
8	—	—	$\bar{1}$·0445	0·0395
9	—	—	$\bar{1}$·0735	0·0410
11	—	Sodium acetate and acetic acid	$\bar{1}$·1101	0·0431
12	—	" "	$\bar{1}$·1030	0·0426
13	—	" "	$\bar{1}$·0186	0·0379
14	—	Sodium bicarbonate	$\bar{1}$·0227	0·0382
15	2·7	" "	$\bar{1}$·0112	0·0387
16	2·7	" "	$\bar{1}$·0514	0·0408
17	2·7	" "	$\bar{1}$·0899	0·0431
18	7·8	Sodium acetate and acetic acid	$\bar{1}$·1339	0·0447
19	7·8	" "	$\bar{1}$·0281	0·0391
20	3·0	" "	$\bar{1}$·0441	0·0404
21	3·0	" "	$\bar{2}$·9904	0·0376
22	3·0	" "	$\bar{1}$·0956	0·0429
23	10·0	" "	$\bar{1}$·0526	0·0413
24	10·0	" "	$\bar{1}$·0894	0·0431
25	10·0	" "	$\bar{2}$·9896	0·0382
26	10·0	" "	$\bar{1}$·0188	0·0392
27	8·5	Sodium bicarbonate	$\bar{1}$·0029	0·0389
28	8·5	" "	$\bar{2}$·9388	0·0360
29	8·5	" "	$\bar{1}$·0663	0·0427
30	8·5	" "	$\bar{1}$·0326	0·0415

When this device was applied to the electrode in the protein solution, the E.M.F. readings showed a gradual fall of 1 to 2 millivolts in the first hour or so, after which they remained practically steady. Sometimes there was a slight drift of about 0·0001 volt per hour.

The whipping device was therefore used in all the observations on solutions 11 to 30. It was also used in the case of No. 9 in the first series, where it was found to make no difference whatever to the steadiness or to the absolute value of the reading in the case of a pure KCl solution.

The reproducibility of the readings obtained on the solutions containing protein is best assessed by plotting the E.M.F. against the logarithms of the known concentrations of chlorine (in mols. per 1000 g. water). This is done in Fig. 1, where the straight line is that given by the preliminary observations. It will be seen that the points for the four solutions (Nos. 11 to 14) containing

no protein practically coincide with this line, showing that the electrode is uninfluenced by the sodium acetate or bicarbonate present. On the other hand, the points for the solutions containing protein (Nos. 15 to 30) all lie above the line, by anything up to 0·0023 volt. This means that the presence of the protein alters the relationship of the E.M.F. to the concentration of chlorine. The discrepancy is not very great, but it seems to be irregular, for the deviation of the points in Fig. 1 from the straight line bears no relation either to the concentration of protein present or to the hydrogen ion concentration (bicarbonate or acetate buffer). So long as this discrepancy is unexplained or cannot be allowed for, the silver-silver chloride electrode, with this technique, cannot be used for the accurate estimation of chlorides in the presence of protein.

Observations on oxalated human plasma.

Haemoglobin-free plasma was obtained by centrifuging oxalated blood obtained from arm veins, and the potentials given by an electrode dipping into this plasma were observed with the technique already described. It was found impossible to get a satisfactorily steady reading. Nine attempts were made. Sometimes the reading showed an irregular drift; sometimes it showed an initial fall, a steady period, and then either a subsequent rise or a subsequent fall; in one case only it remained steady for a reasonable period (from the 97th to the 292nd minute). Moreover, if two sets of readings were made on different portions of the same specimen of plasma, at an interval of several hours or 1 day, they were found to be completely different. The unsteadiness of the readings, and this utter lack of reproducibility, are illustrated by the following figures:

(*a*) Fresh plasma		(*b*) Further quantity of same plasma 3 hours later; same electrode	
Time (minutes)	Reading (volts)	Time (minutes)	Reading (volts)
0	Liquid junction made	2	0·0501
3	0·0403	8	0·0525
10	0·0412	20	0·0530
24	0·0424	31	0·0515
37	0·0420	44	0·0500
50	0·0416	55	0·0482
63	0·0413	65	0·0469
99	0·0412	74	0·0462
136	0·0411	97	0·0452
154	0·0411	120	0·0447
		209	0·0440
		236	0·0438
		262	0·0436

When potentials were determined on successive days on the same plasma using a fresh electrode for each occasion an increase of 7 millivolts was observed on the second day. This would suggest that changes occur in the plasma with time which affect the activity of the chlorine ion. On the other hand, electrodes which had been immersed in plasma for 2 to $5\frac{1}{2}$ hours gave, after washing, values of E in a pure solution of KCl which differed by 5 to 7 millivolts from those recorded before treatment with plasma. This is evidence that plasma has a definite "poisoning" effect on the electrode.

Discussion.

The observations on pure KCl solutions. It is interesting to note that the E.M.F. in the case of No. 9 solution, in which the electrode was kept vibrating, is in conformity with those obtained in the other solutions where the electrode was still; this throws doubt on Brown and Hill's [1921] conclusion that in such measurements as these the liquid must be kept still.

The observations on KCl solutions containing protein gave E.M.F.'s which are all higher than was expected. The solutions appear to contain a greater concentration of Cl′ than is known to be present (or else the activity coefficient of the ion is raised). Possible explanations of this are as follows.

1. There may be a disparity in the concentrations of ions between the solution inside the protein micellae and the solution surrounding them. Since the proteins are on the alkaline side of their isoelectric point, the concentration of chlorine outside the micellae (and therefore affecting the electrode) might be expected to be greater than that inside, and therefore greater than the average chlorine concentration of the whole solution. If this were the explanation, however, the apparent increase in chlorine concentration as judged from the E.M.F. should bear some relation to the concentration of protein present and to the hydrogen ion concentration; no such relation appears in the readings that were obtained.

2. The protein molecules or ions may be hydrated. Again, if this were the explanation, the apparent increase in chlorine concentration should be related to the protein concentration present. Moreover, it has been shown by Corran and Lewis [1922], in the case of sucrose, which is known to be hydrated in solution, that the activity of chlorine in the presence of sucrose is the same as in its absence, the concentrations being calculated in the total water present (free water plus water of hydration).

3. The behaviour of the electrode may be modified by the presence of protein. Oryng and Pauli [1915], using calomel electrodes, found a lower E.M.F. with salt-free dialysed serum-protein than with distilled water; in the chain they used this meant that serum-protein after being shaken with calomel appeared to have a higher concentration of chlorine than water shaken with calomel. In the light of other experiments they attributed this to the formation and ionisation of a mercury-protein complex, and in subsequent work they introduced a correction for this effect, assuming it to be proportional to the concentration of protein present. Warburg [1922] criticises this. He lays down two conditions theoretically necessary for the determination of Cl′ electrometrically:

(*a*) that the Hg or Ag ion concentration be determined solely by the activity of the Cl′; this is not the case if metal-protein complexes be formed;

(*b*) that none of the other solutes be affected by the Hg or Ag ions.

He considers that in the presence of protein neither of these conditions is fulfilled, and the determination is impossible.

On the whole, the third of these suggested explanations seems to carry most weight. In the absence of further positive findings, the results that have been given provide an experimental justification for the criticism that Warburg made on purely theoretical grounds.

The observations on oxalated plasma show that in whole plasma there are more disturbing factors at work than simply the presence of protein, and the E.M.F. obtained are quite unreliable as a measure of the chlorine present. What constituent of plasma is responsible for the "poisoning" effect described was not determined, for in view of the unsatisfactory results obtained with the protein solutions it seems very unlikely that the electrometric method can be applied with success to the estimation of chlorine in plasma.

SUMMARY.

Chlorine ion potentials were measured by means of silver-silver chloride electrodes. The readings obtained in pure potassium chloride solutions were steady and reproducible. The readings obtained in potassium chloride solutions containing serum-protein were steady, but not accurately reproducible, and indicated a higher concentration of chlorine ions than was known to be present. The readings obtained in oxalated human plasma were not steady, and were not reproducible. Immersion in plasma "poisoned" the electrode. The technique as used is not applicable to the estimation of the chlorine ion in plasma.

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CLV. THE DETERMINATION OF CARNOSINE.

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(*Received July 30th, 1928.*)

Early in the year 1921 Clifford published a method of estimating carnosine, which is based on the colour produced when carnosine reacts with diazobenzene-sulphonic acid. The resultant coloured solution is matched in a colorimeter against a standardised mixture of Congo red and methyl orange [Koessler and Hanke, 1919].

The method was extended to the estimation of chromogenic substances in muscle (mainly, no doubt, carnosine). The muscle, in this process, is minced, weighed, and extracted with water, the proteins in the extract precipitated by metaphosphoric acid, and the filtrate treated as if it were a carnosine solution. Based on this method a series of papers has been published [Clifford, 1921, 1, 2, 1922, 1, 2, 1923] with results which are consistent with each other, and which form a starting-point for the investigation of the functions of carnosine.

Unfortunately the other two workers who have concerned themselves with carnosine estimations do not agree with Clifford in some, and those the simplest and most striking, of her findings. G. Hunter has sorely criticised many details of the method [Hunter, 1921, 1922, 1924] and even suggests that the carnosine nitrate upon which Clifford's estimations are based was only 43 % pure. As this was supplied to her by Dr H. H. Dale as pure, and as the melting-point was correct, it is a suggestion not easily accepted. Mitsuda [1923] agrees with Hunter in saying that the carnosine content of cats' muscle varies.

Now this disagreement is intolerable, and it was resolved to re-examine some of the salient points of difference. The experimental work has been shared, so that one of us (V. H. M.) was able to hand over to the other (W. M. C.) clear, colourless solutions for estimation, the source, previous history, and carnosine content of which were quite unknown to the estimator.

Method of estimation.

(1) *On solutions of pure carnosine.*

The method follows that of Koessler and Hanke [1919]. To 10 cc. of 1·1 % sodium carbonate solution are added 2–x cc. of distilled water, 4 cc. of the diazo-reagent (kept cool by ice in hot weather) and x cc. of the solution

to be estimated. The volume of the last naturally varies inversely as the strength of the solution. The mixed solutions are left standing at room temperature for 3–5 minutes to develop the full colour. Then the colour is estimated in a Duboscq colorimeter with Koessler and Hanke's C.R.–M.O. standard mixture as a comparison. No deviations from the Koessler and Hanke technique have been adopted except that the minute directions as regards time of mixing the reagents have not been found necessary, and the strength of the indicator chosen is such that a match at 30 mm. with the carnosine solution set at 20 mm. corresponds to 0·0001 g. of carnosine.

(2) *On tissues containing iminazole compounds.*

The tissue is minced, and from 1 to 6 g. are weighed accurately into a small Monax beaker. From 80 to 90 cc. of distilled water are added, and the whole brought slowly to the boil. 5 cc. of a 20 % solution of metaphosphoric acid are then added, and the mixture is allowed to stand (best overnight). The mixture is filtered and the precipitate washed. The filtrate is made up to 100 cc. Of this crystal clear fluid about 1 cc. is used for the estimation and the technique is exactly that of the preceding paragraph.

Reinvestigation of the method.

(1) *On pure carnosine.*

(*a*) *Accuracy of the method.* As the original work was carried out in the difficult post-war period when dyes for the C.R.–M.O. indicator were hard to obtain, and as the original sample of carnosine nitrate, guaranteed pure as it was by Dr Dale, was rather small, it appeared to us necessary to re-standardise the old indicator (the stock solutions of which are still in use) with a new pure sample of carnosine.

Attempts to make in this laboratory, or to purchase on the market, samples of carnosine having proved abortive, this crucial test could not have been made if it had not been for the kindness of Dr Dudley, to whom we are indebted for half a gram of the pure base carnosine.

Solutions of known strength were made in distilled water from this sample and estimated, with the results shown in Table I. Carefully calibrated pipettes, measuring flasks and burettes were used.

Table I.

Solution	Calculated %	Found %	Difference g.
1	0·1460	0·146 (5)	+0·0005
2	0·0730	0·073	Nil
3	0·0487	0·050	+0·0063
4	0·0365	0·036 (6)	+0·0001
5	0·3595	0·359	−0·0005
6	0·2200	0·219	−0·0010
7	0·6190	0·617	−0·0020
8	0·3650	0·365	Nil

The figures speak for themselves. With one exception (Solution 3), the agreement is remarkably close, and in this case there is a note in the protocol (V. H. M.) that the emptying of the automatic pipette did not appear satisfactory. Neglecting this one solution, the agreement is little short of perfect. There seemed to be no reason for going any further with this part of the work, which indicates (i) that the original carnosine was not, as has been suggested, 43 % pure only, but chemically pure; (ii) that the stock solutions of dyes keep perfectly satisfactorily over a number of years; and (iii) that the method of estimation has a high order of accuracy.

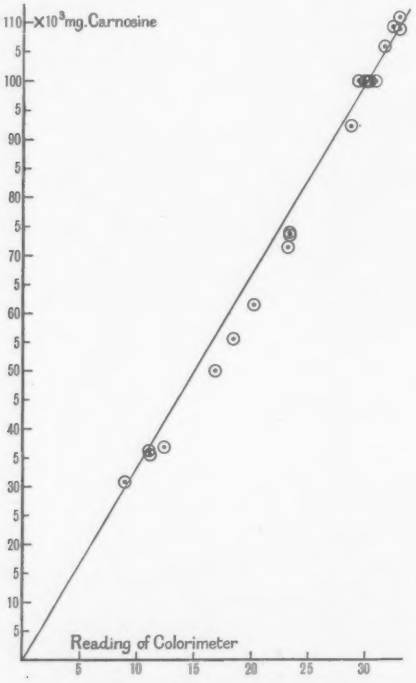

Fig. 1. Readings on the colorimeter plotted against carnosine content of solution. The straight line represents the true linear relation.

(*b*) *Linear agreement between colour and amount of carnosine.* It has been stated [Clifford, 1921, 1] that the production of diazo-colour is not directly proportional to the amount of carnosine present in the solution to be estimated, whereas Hunter [1921] claims a complete proportionality. Mitsuda [1923], while agreeing with Clifford that the relation is not linear, states that the deviation from linearity is not so great as Clifford found.

In making the estimations given in Table I some 32 adjustments of the strengths of the solutions were made before the end-point was reached. Fortunately, accurate colorimeter readings were taken of the matches given before the correct dilutions were obtained, so that we have a large number of readings comparing colour produced with the known amounts of carnosine present. These are given in Fig. 1. It will be seen that there is a fair approximation to linearity. None the less, the best and most normally displaced from the mean are those when the reading of the C.R.–M.O. in the colorimeter is at 30 mm. and the unknown is set at 20 mm., the conditions under which estimations are usually made.

(2) *On muscle extracts.*

The chief interest of a method of estimating carnosine lies in its applicability to tissues. There are often large amounts in vertebrate muscle of substances which give the diazo-reaction. The iminazole compounds present are carnosine, purine compounds, histamine and possibly histidine; but no one doubts that it is carnosine which is responsible for the major part of the colour. Hunter [1922] estimates it at 95 % of the total, so that an accurate and consistent method of estimating the chromogenic substances in muscle is likely to throw some light upon the functions of carnosine.

Now one thing which appeared certain from Clifford's work [1921, 2; 1922, 2] on the distribution of carnosine in animal tissues, is that all the skeletal muscles of any animal of any one species have an almost constant amount of carnosine, but that each species has its own constant which may range from zero (*e.g.* cod, sole, finch) to 1 % (*e.g.* ox).

Both Hunter and Mitsuda maintain, on the other hand, that there is no constancy in the carnosine content of different animals of the same species. Estimates are given for the gastrocnemius of the cat which run from 0·007 % to 1·293 %, while Clifford's figures lie between 0·48 % and 0·51 %.

To eliminate the personal factor in the colour comparisons a large series of estimations of the chromogenic substances in ox and cat muscle has been made, using 13 different muscles or groups of muscle in the ox and 9 different muscles and 4 groups of muscle in the cat. In all these estimations except the last four, by which time it was considered proved that the personal factor did not count at all, the weighings and the extractions of the muscle were made by one of us (V. H. M.) and nothing but clear solutions handed over to the other (W. M. C.) for estimation.

Bullocks' muscle is a convenient tissue to investigate, but it must be freshly killed and not imported [Clifford, 1922, 1]. Various "cuts" were bought on various dates and the chromogenic substances found are expressed as carnosine in Table II. Each figure given is the mean of at least three parallel estimations agreeing within 1 %—indeed the greatest difference between highest and lowest estimate of any one sample is 8 parts in 970.

Table II. *Chromogenic substances in bullocks' muscle expressed as carnosine.*

"Cut"	Carnosine %
Rump steak	0·983
Shoulder steak	0·990
Fillet steak	0·981
Flank	0·981
Silver side	0·974
Shin	0·981
Skirt	0·975

It is obvious that there is great constancy in the amount. The figures show that the amount of carnosine is constant from animal to animal and for different groups of muscle. To get discrete muscles and not "cuts," five pairs and an odd one were dissected from a bullock's head. Their analyses are given in Table III.

Table III. *Chromogenic substances in muscles of bullock's head expressed as carnosine.*

Name of muscle	Carnosine %
Superior constrictor (?)	0·987, 0·991
Masseter	0·986
Buccinator	0·994, 0·991
Trapezius	0·991, 0·986
Pterygoideus externus	0·992, 0·990
„ internus	0·990, 0·991

It thus appears from the results in Tables II and III that the carnosine content of the different skeletal muscles of the ox is approximately constant both from muscle to muscle and from animal to animal. The greatest variation is from 0·975 % to 0·994 %—at the most a little under 2 %.

Table IV. *Chromogenic substances in cats' muscles expressed as carnosine.*

No. of Exp.	Name of muscle	Carnosine %	Notes
1	Gastrocnemius	0·500, 0·496	
	Psoas	0·499, 0·496	
	Gluteus max. (right)	0·490	
	„ (left)	0·492	
	Soleus	0·483	
	Tensor fasciae	0·485	
	Triceps brachialis	0·484, 0·483	
	Semi-membranosus	0·495, 0·498	
	Quadriceps	0·480, 0·482	
	Biceps femoris	0·498, 0·500	
2	—	0·517, 0·509, 0·515	Killed 10. iii. 28. In cold room till 12. iii. 28. Muscle in (4) smells of ether
3	—	0·511, 0·513, 0·514	
4	—	0·484, 0·477, 0·485	
5	—	0·482, 0·487, 0·480	Killed 12. iii. 28. Sent that day. Smells slightly of ether

It is, however, mainly with the cat that Hunter and Mitsuda claim to have found differences in carnosine content between muscle and muscle and cat and cat, though they state that the right gastrocnemius has the same amount as the left. A careful dissection of one cat yielded nine muscles in reasonable amounts for estimation, and we obtained, in addition, from the Medical Research Institute, through the kindness of Dr Dale, four carcases of

cats which had been used for experiments there. From these, portions of muscle were taken, but no careful dissection into separate muscles was made. The results are given in Table IV.

It is hardly necessary to comment on the figures of Table IV. No case can be made from them for a wide variation of the carnosine content of skeletal muscle from muscle to muscle, or from animal to animal.

The difference between the highest and the lowest is but 8 % of the lowest, whereas Mitsuda's figures run (for the gastrocnemius) from 0·684 % to 1·293 % and Hunter's from 0·007 % to 0·433 %.

Discussion.

The results detailed above, though going far to substantiate the adequacy and accuracy of Clifford's method for the estimation of carnosine, do little to clear up the confusion. This method has been used with great accuracy to determine the amount of carnosine in solutions of strength known to one person but not to the estimator. Further, the carnosine content of various solutions, colourless and giving no clue as to their content on inspection, has been shown to be proportional to the amount of tissue from which they came. Care was taken by the one who carried out the weighings that there should be great differences in the weight of tissue used, whereas the final solution handed over to the estimator was always 100 cc. The weight of tissue was varied 600 % without any effect on the final result. Consequently we consider that these figures make out a good case for the estimation of carnosine by Clifford's method, though we admit that we cannot understand or explain why other workers get such discordant results.

Summary.

1. A reinvestigation of Clifford's method of estimation of carnosine shows that the original carnosine upon which the work was based was pure and that the indicators, once standardised, were satisfactory.

2. By this method the carnosine content of a solution of pure carnosine in water can be estimated with an error less than 1 %.

3. Using this method to estimate the chromogenic substances in samples of muscle, it has been shown that they vary in amount directly as the amount of muscle taken, no matter what skeletal muscle is used, or if it be from a different animal of the same species. In other words, for the skeletal muscle of a species the carnosine content is a constant. This constant varies from species to species though not from member to member of that species.

4. This work on ox and cat muscle confirms observations on the muscles of rats, rabbits, calves, sheep and lambs [Clifford, 1921, 2].

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CLVI. NEUTRAL SALT ADDITION COMPOUNDS OF *N*-METHYLATED GLYCINES: THEIR FORMULATION AND THAT OF THEIR HYDRATES.

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(*Received August 31st, 1928.*)

It is now a well-established fact that some of the naturally occurring α-amino-acids have the property of forming addition compounds with neutral salts, such as the halides of the alkali and alkaline earth metals. It is probable that all exhibit this property to some extent in solution, but the isolation of crystalline additive products is only successful where the solubility of the addition product is less than that of the amino-acid or the inorganic salt, a condition of infrequent occurrence.

Glycine, $NH_2.CH_2.CO_2H$, furnishes the best example of an extended series of addition compounds [Pfeiffer and Modelski, 1912, 1913; King and Palmer, 1920] and its simple derivatives sarcosine, $NHMe.CH_2.CO_2H$, alanine, $NH_2.CHMe.CO_2H$, and betaine, $\overset{+}{N}Me_3.CH_2.\overset{-}{C}O_2$, have supplied a few further instances of this additive property [Pfeiffer, 1922].

The aim of the present investigation was primarily the use of *N*-dimethyl-aminoacetic acid, $NMe_2.CH_2.CO_2H$, where the glycine type is retained, but where there are no longer any hydrogen atoms attached to nitrogen. This acid has given a particularly complete series of additive compounds with the chlorides, bromides and iodides of calcium, strontium, barium, lithium and sodium, but only one compound with a potassium halide. The composition of these complexes when compared with those, so far as they were known, of glycine, sarcosine and betaine revealed such striking analogies that the preparation of the missing additive compounds of sarcosine and betaine was also undertaken.

The complete series of additive compounds of glycine and its mono-, di-, and tri-methyl-derivatives is shown in the following table where G = glycine, S = sarcosine, D = dimethylglycine and B = betaine (anhydrous).

With one exception, all of these compounds belong to one of four types. They contain a metallic salt associated with 1, 2, 3 or 4 molecules of the amino-acid and for such there is only one consistent interpretation of their structure. The inorganic components are highly ionised salts and there is

Glycine	Sarcosine	Dimethylglycine	Betaine
$G.CaCl_2.3H_2O$[a]	$S.CaCl_2.4H_2O$[b]	$D.CaCl_2.4H_2O$[e]	—
$G_4.CaCl_2.4H_2O$[a, c, d]	—	—	—
$G_3.CaCl_2$[a]	$S_3.CaCl_2$[b]	—	—
$G_2.CaBr_2.4H_2O$[a, d]	—	$D_2.CaBr_2.6H_2O$[e]	—
$G_2.CaBr_2$[d]	$S_3.CaBr_2$[e]	—	—
$G_2.CaI_2.3H_2O$[c, d]	$S_2.CaI_2.3H_2O$[e]	$D.CaI_2$[e]	$B_2.(CaI_2)_2.11H_2O$[e]
G_4CaI_2[d]	—	—	—
$G_2.SrCl_2.H_2O$[c]	$S.SrCl_2.4H_2O$[b, e]	$D.SrCl_2.4H_2O$[e]	$B.SrCl_2.4H_2O$[e]
$G_2.SrCl_2.3H_2O$[a]	—	—	—
$G_2.SrBr_2.3H_2O$[a]	$S.SrBr_2.4H_2O$[e]	$D.SrBr_2.4H_2O$[e]	$B.SrBr_2.5H_2O$[e]
$G_4.SrI_2$[d]	$S_2.SrI_2.2H_2O$[d]	$D_2.SrI_2.3H_2O$[e]	$B_2.SrI_2.4H_2O$[e]
—	$S.SrI_2.4H_2O$[e]	—	—
$G_2.BaCl_2.H_2O$[a, c]	$S.BaCl_2.4H_2O$[b]	$D.BaCl_2.4H_2O$[e]	$B.BaCl_2.4H_2O$[a]
—	$S.BaBr_2.4H_2O$[b]	$D.BaBr_2.4H_2O$[e]	$B.BaBr_2.4H_2O$[a]
$G_2.BaBr_2.H_2O$[a]	—	—	$B_2.BaBr_2.6H_2O$[b]
$G_4.BaI_2$[d]	$S_2.BaI_2.2H_2O$[e]	$D_2.BaI_2.4H_2O$[e]	$B.BaI_2.4H_2O$[e]
$G.LiCl.H_2O$[a, c]	$S.LiCl.H_2O$[d, e]	$D.LiCl.2H_2O$[e]	—
$G_2.LiCl.H_2O$[a]	—	—	—
$G.LiBr.H_2O$[a, c]	$S.LiBr.H_2O$[d, e]	$D.LiBr.H_2O$[e]	$B.LiBr.2\frac{1}{2}H_2O$[e]
$G_2.LiBr.H_2O$[a, c]	—	$D_3.LiBr$[e]	—
$G.LiI.H_2O$[d]	$S.LiI.1\frac{1}{2}H_2O$[d, e]	$D.LiI.6H_2O$[e]	$B_2.LiI.H_2O$[e]
—	$S.NaCl.H_2O$[d]	$D.NaCl.2H_2O$[e]	$B.NaCl.1\frac{1}{2}H_2O$[e]
$G.NaBr.1\frac{1}{2}H_2O$[d]	$S.NaBr.H_2O$[d]	$D.NaBr.H_2O$[e]	—
$G_2.NaBr.H_2O$[c]	—	$D_2.NaBr.2H_2O$[e]	$B_2.NaBr.1\frac{1}{2}H_2O$[e]
$G_2.NaI.H_2O$[c, d]	$S.NaI.H_2O$[b, d]	$D.NaI.3H_2O$[e]	$B.NaI.3H_2O$[e]
—	$S_3.KCl.5H_2O$[e]	—	—
—	$S_4.KBr.4H_2O$[b, e]	—	$B.KBr.2H_2O$[a]
$G_4.KI_3$[d]	$S_3.KI$[d]	$D.KI.1\frac{1}{2}H_2O$[e]	$B.KI.2H_2O$[a]
—	$S_4.KI.4H_2O$[b]	—	$B_2.KI.2H_2O$[a]

[a] Pfeiffer and Modelski [1912].
[b] Pfeiffer and Wittka [1915].
[c] King and Palmer [1920].
[d] Pfeiffer, Klossmann and Angern [1924].
[e] Anslow and King (this paper).

little doubt that they remain so in these complexes and are held by electro-valencies. The metallic cations are free to co-ordinate with the carboxyl oxygen atoms of the amino-acid molecules, two of the four electrons of the carbonyl oxygen atom entering the outer shell of the metallic cation in the manner suggested for the neutral salt addition compounds of alkaline earth glutamates and aspartates by Anslow and King [1927]. The compounds with sarcosine or glycine, for instance, where all four types are observed will have the general structure

$$[(NR_1R_2.CH_2.C(OH):O)_{1-4}M]\,X_{1-2},$$

where M is the metallic cation co-ordinated through carbonyl oxygen to 1, 2, 3 or 4 amino-acid molecules and X is the halogen ion, the number, 1 or 2, depending on the electro-valency of M. On no other formulation, such for example as the combination of the halogen ion with the ammonium form of the amino-acid, does it seem possible to interpret such complexes as triglycine calcium bromide prepared by Pfeiffer, Klossmann and Angern [1924] or its sarcosine analogue, trisarcosine calcium bromide prepared by us.

So far, the formulation of the amino-acid molecules in their relation to the metallic atoms has been discussed to the exclusion of the water molecules.

The electronic arrangement of the oxygen atom in water is, however, similar to that of the carbonyl oxygen atom and the composition of the addition compounds formed between dimethylglycine and lithium bromide, $D.LiBr.H_2O$ and $D_2.LiBr$, and between sarcosine and the potassium halides, $S_3.KCl.5H_2O$, $S_4.KBr.4H_2O$ and $S_4.KI.4H_2O$, even suggests inter-equivalence in these cases of amino-acid and water molecules. If, therefore, the view be accepted that these additive compounds are held together by co-valencies, then in a series of about eighty compounds such as are enumerated here, one might by a consideration of the number of water molecules expect to find examples where the known maximum co-ordination number of the metallic element is exhibited. Such indeed is the case. Consider, for example, the additive compounds of dimethylglycine. The majority of the alkaline earth halide addition compounds are hydrated and most of them may be formulated as having a co-ordination number of 6. The commonest type is $D.MX_2.4H_2O$ and this falls into the scheme if each oxygen atom of the carboxyl group occupies one position in the co-ordination complex. Thus, dimethylglycine calcium chloride tetrahydrate would be written

$$\left[NMe_2.CH_2.C\begin{matrix}O\\ \\O\\H\end{matrix}Ca\vdots(OH_2)_4 \right]Cl_2$$

whilst the type exemplified by di-dimethylglycine barium iodide tetrahydrate would be written

$$\left[(NMe_2.CH_2.C(OH):O)_2\ Ba\vdots(OH_2)_4\right]I_2.$$

The exceptional hexahydration observed with di-dimethylglycine calcium bromide hexahydrate suggests either a co-ordination number of 8 for calcium as postulated by Pfeiffer in $[Ca(NH_3)_8]Cl_2$ or attachment of the extra water molecules to the tertiary nitrogen atoms, one to each.

In a similar way the hydrates of the alkali halide addition compounds of dimethylglycine, with the exception of that with lithium iodide, may be formulated on the basis of a co-ordination number of 2 or 4 for the alkali metal. The same principles apply to the majority of the compounds of glycine, sarcosine and betaine.

One or two cases call for further comment. The exceptional series of compounds formed between sarcosine and the potassium halides mentioned above are most simply interpreted on a co-ordination number of 8 for potassium, the interchange of water and amino-acid molecules, and the odd number of amino-acid and water molecules in $S_3.KCl.5H_2O$, being difficult to account for in any other way.

If the above views are correct, they ought to account for the most remarkable compound of this series yet observed. Betaine and calcium iodide form a well-authenticated addition compound containing eleven molecules of

water, $B_5.(CaI_2)_2.11H_2O$. It crystallises in the cubic system and is optically isotropic. This is formulated as

$$\left[(H_2O)_3 \vdots \overset{\overset{B}{\vdots}}{\underset{\underset{B}{\vdots}}{Ca}} \cdot B \cdot \overset{\overset{B}{\vdots}}{\underset{\underset{B}{\vdots}}{Ca}} \vdots (OH_2)_3\right] I_4$$

where B is the hydrated form of betaine and calcium has the usual co-ordination number of 6. The use of betaine as a bridge between the two calcium atoms involves the use of both oxygen atoms of the carboxyl group, each occupying one co-ordination position of a calcium atom, and thus strengthens the formulation proposed above for the commonest type of compound observed between dimethylglycine and the alkaline earth halides, $D.MX_2.4H_2O$.

Experimental.

Addition compounds of N-*methylaminoacetic acid* (*sarcosine*).

Tri-sarcosine calcium bromide. Sarcosine hydrobromide (4·25 g.) was boiled with excess of calcium hydroxide; after treatment with carbon dioxide the solution was boiled, filtered and concentrated. On keeping, large, well-formed, rectangular plates were obtained (1·0 g.). These were filtered off rapidly, transferred to porous plate and well pressed to remove all adhering mother-liquor. (This method was used for all the addition compounds.)

0·2086 g.; 0·1717 g. AgBr. Br = 35·0 %.
0·1902 g.; NH_3 = 14·48 cc. *N*/10 acid. N = 8·5 %.
Calc. for $(C_3H_7O_2N)_3.CaBr_2$; Br = 34·3 %; N = 9·0 %.

Di-sarcosine calcium iodide trihydrate. Square plates (1·9 g.) were obtained in the usual way from sarcosine hydriodide (4·3 g.) and excess calcium hydroxide. (Found: I, 48·1; N, 6·7 %; whence I : N = 1 : 1·26.) Recrystallisation gave elongated rectangular plates (0·5 g.) of the pure compound.

0·1720 g.; 0·1540 g. AgI. I = 48·4 %.
0·1740 g.; NH_3 = 6·45 cc. *N*/10 acid. N = 5·2 %.
Calc. for $(C_3H_7O_2N)_2.CaI_2.3H_2O$; I = 48·2 %; N = 5·3 %.

Di-sarcosine barium iodide dihydrate. Prepared in a similar way to the previous compound, this *dihydrate* crystallised in needles (0·9 g.).

0·2090 g.; 0·1607 g. AgI. I = 41·6 %.
0·2070 g.; NH_3 = 6·53 cc. *N*/10 acid. N = 4·4 %.
Calc. for $(C_3H_7O_2N)_2.BaI_2.2H_2O$; I = 41·9 %; N = 4·6 %.

Sarcosine strontium chloride tetrahydrate. This compound, previously prepared by Pfeiffer and Wittka [1915] from equimolecular solution, was obtained in needles from a solution of sarcosine and strontium chloride in the ratio 2 : 1. (Found: Cl, 21·7; N, 4·4 %. Theory: Cl, 22·2; N, 4·4 %.)

Sarcosine strontium bromide tetrahydrate. Needles (1·4 g.) were obtained in the usual manner.

0·2006 g. dried at 120° lost 0·0336 g. H_2O = 16·8 %.
0·2006 g.; 0·1810 g. AgBr. Br = 38·4 %.
0·2039 g.; NH_3 = 5·36 cc. *N*/10 acid. N = 3·7 %.
Calc. for $C_3H_7O_2N.SrBr_2.4H_2O$; H_2O = 17·6 %; Br = 39·1 %; N = 3·4 %.

Sarcosine strontium iodide tetrahydrate. Concentration of a solution prepared in the usual way gave needles (1·2 g.) which were impure. (Found: I, 48·2; N, 3·3 %; whence I : N = 1 : 0·62.) When, however, an equimolecular solution was concentrated needles (1·3 g.) of the pure compound were obtained.

0·2022 g.; 0·1872 g. AgI. I = 50·0 %.
0·2082 g.; NH_3 = 4·10 cc. *N*/10 acid. N = 2·8 %.
Calc. for $C_3H_7O_2N.SrI_2.4H_2O$; I = 50·5 %; N = 2·8 %.

Tri-sarcosine potassium chloride pentahydrate. Sarcosine hydrochloride (4·2 g.) was dissolved in 33·3 cc. *N* potassium hydroxide and the solution concentrated. After the removal of potassium chloride (1·1 g.), well-formed hexagonal plates were deposited (0·4 g.).

0·2080 g.; 0·0661 g. AgCl. Cl = 7·9 %.
0·1885 g.; NH_3 = 13·04 cc. *N*/10 acid. N = 9·7 %.
Calc. for $(C_3H_7O_2N)_3.KCl.5H_2O$; Cl = 8·2 %; N = 9·7 %.

The existence of the tetra-sarcosine potassium bromide tetrahydrate of Pfeiffer and Wittka [1915] and of sarcosine lithium chloride monohydrate, sarcosine lithium bromide monohydrate and sarcosine lithium iodide sesquihydrate of Pfeiffer, Klossmann and Angern [1924] was confirmed.

Salts of N-*dimethylaminoacetic acid.*

N-*Dimethylaminoacetic acid hydrochloride.* This compound, described by Johnson [1906] and Friedmann [1908], was prepared by slowly dropping an aqueous solution of monochloroacetic acid (8·25 g. in 8·2 cc.) into a solution of dimethylamine (100 cc., 16·5 % aq.) cooled to 0°, followed by four hours' heating in a pressure bottle at 55°. An excess of baryta was added on cooling, and the liberated dimethylamine removed by concentration *in vacuo*. After exact precipitation of the barium by sulphuric acid and removal of the barium sulphate, concentration to a small volume (at 50°), followed by the addition of an equal volume of alcohol, gave a mass of transparent tablets, melting at 189–191°. The total yield was 8·75 g. (Found: Cl = 25·6 %. Theory: 25·2 %.)

The free acid was prepared by adding to a solution of the hydrochloride a slight excess of silver carbonate; after removal of the excess of silver with hydrogen sulphide, concentration gave very hygroscopic tablets which were dried *in vacuo* over sulphuric acid. This acid is readily soluble in dry methyl alcohol, but crystallises well in sheaves of needles on careful addition of acetone. M.P. 176–178° (Johnson gives M.P. 157–160°). (Found: N = 13·3 %. Theory: 13·6 %.)

The *chloroaurate* is dimorphous and very readily soluble in water. The less stable form separates as a microcrystalline pale yellow powder on concentration and in contact with the solution soon changes into deep orange rhombs. M.P. 96–98°.

0·1131 g. dried at 90° lost 0·0039 g. H_2O = 3·4 %.
0·1399 g.; 0·0587 g. Au. Au = 42·0 %.
Calc. for $C_4H_9O_2N.HAuCl_4.H_2O$; H_2O = 3·9 %; Au = 42·8 %.

N-*Dimethylaminoacetic acid hydrobromide.* Prepared by concentrating a solution of the amino-acid containing excess hydrobromic acid, this salt was obtained in anhydrous, elongated hexagonal plates which are slightly hygroscopic; it is less soluble than the hydrochloride. M.P. 158–160°.

0·2013 g.; 0·2053 g. AgBr. Br = 43·4 %.
Calc. for $C_4H_9O_2N.HBr$; Br = 43·4 %.

N-*Dimethylaminoacetic acid hydriodides.* The *monohydriodide*, prepared in a similar way to the hydrobromide, crystallises in hexagonal plates which are anhydrous and melt at 149°. Solutions become coloured on heating.

0·1964 g.; 0·1981 g. AgI. I = 54·5 %.
Calc. for $C_4H_9O_2N.HI$; I = 54·9 %.

The *semi-hydriodide*, prepared by using less hydriodic acid, concentrating to a small volume and adding alcohol until turbid, crystallised on standing overnight in tufts of broad needles. The recrystallised compound (a *monohydrate*) melts at 157°, but is decomposed if heated to 120° for half an hour.

0·2017 g.; 0·1326 g. AgI. I = 35·5 %.
0·2040 g.; NH_3 = 11·94 cc. *N*/10 acid. N = 8·2 %.
Calc. for $(C_4H_9O_2N)_2.HI.H_2O$; I = 36·1 %; N = 8·0 %.

Attempts to prepare the semi-hydrochloride and semi-hydrobromide were unsuccessful—the less soluble normal salts contaminating the products obtained in each case, even after recrystallisation.

Addition compounds of N-*dimethylaminoacetic acid.*

N-*Dimethylaminoacetic acid calcium chloride tetrahydrate.* *N*-Dimethylaminoacetic acid hydrochloride (4·8 g.) dissolved in a small volume of water was boiled with excess of calcium hydroxide. Carbon dioxide was passed to remove the excess, and the solution was boiled to decompose carbamates, filtered and concentrated. After standing several days the syrupy solution deposited prismatic rods (1·25 g.).

0·2103 g. dried at 120° lost 0·0502 g. H_2O = 23·9 %.
0·2103 g.; 0·2050 g. AgCl. Cl = 24·1 %.
0·1951 g.; NH_3 = 7·03 cc. *N*/10 acid. N = 5·0 %.
Calc. for $C_4H_9O_2N.CaCl_2.4H_2O$; H_2O = 25·1 %; Cl = 24·8 %; N = 4·9 %.

Di-(N-*dimethylaminoacetic acid*) *calcium bromide hexahydrate.* *N*-Dimethylaminoacetic acid hydrobromide (6·1 g.) was boiled with excess calcium hydroxide in a similar way to the previous compound; 0·75 g. of long rods were obtained. This hexahydrate on drying loses $4H_2O$ at 120° and a further $\frac{1}{2}H_2O$ at 160°.

0·1945 g. dried at 120° lost 0·0271 g. H_2O = 13·9 %.
0·1500 g. dried at 160° lost 0·0239 g. H_2O = 15·9 %.
0·1945 g.; 0·1445 g. AgBr. Br = 31·6 %.
0·1993 g.; NH_3 = 7·43 cc. *N*/10 acid. N = 5·2 %.
Calc. for $(C_4H_9O_2N)_2.CaBr_2.6H_2O$; $4H_2O$ = 14·0 %; $4\frac{1}{2}H_2O$ = 15·8 %; Br = 31·1 %; N = 5·4 %.

A second less pure crop was obtained crystallising in needles.

N-*Dimethylaminoacetic acid calcium iodide.* *N*-Dimethylaminoacetic acid hydriodide (5·8 g.) was boiled with excess calcium hydroxide, and treated in the usual way; the solution deposited long needles (0·7 g.).

0·1937 g.; 0·1692 g. AgI. I = 47·2 %.
0·2022 g.; NH_3 = 7·40 cc. *N*/10 acid. N = 5·1 %.
Calc. for $C_4H_9O_2N.CaI_2$; I = 47·0 %; N = 5·2 %.

N-*Dimethylaminoacetic acid barium chloride tetrahydrate.* Prepared under the foregoing conditions, long needles (0·9 g.) were obtained.

0·2003 g. dried at 120° lost 0·0391 g. H_2O = 19·3 %.
0·2003 g.; 0·1464 g. AgCl. Cl = 18·1 %.
0·2057 g.; NH_3 = 5·78 cc. *N*/10 acid. N = 3·9 %.
Calc. for $C_4H_9O_2N.BaCl_2.4H_2O$; H_2O = 18·8 %; Cl = 18·5 %; N = 3·7 %.

A further crop was obtained on concentration.

N-*Dimethylaminoacetic acid barium bromide tetrahydrate.* Long needles (2·0 g.) were obtained under similar conditions.

0·2079 g. dried at 120° lost 0·0315 g. H_2O = 15·2 %.
0·2079 g.; 0·1634 g. AgBr. Br = 33·5 %.
0·2017 g.; NH_3 = 4·61 cc. *N*/10 acid. N = 3·2 %.
Calc. for $C_4H_9O_2N.BaBr_2.4H_2O$; H_2O = 15·3 %; Br = 33·8 %; N = 3·0 %.

Di-(N-*dimethylaminoacetic acid*) *barium iodide tetrahydrate.* This compound was obtained in needles (0·6 g.) after recrystallisation of a slightly impure crop (1·6 g.) obtained in the usual way.

0·1943 g. dried at 120° lost 0·0186 g. H_2O = 9·6 %.
0·1943 g.; 0·1338 g. AgI. I = 37·2 %.
0·1969 g.; NH_3 = 5·32 cc. *N*/10 acid. N = 3·8 %.
Calc. for $(C_4H_9O_2N)_2.BaI_2.4H_2O$; H_2O = 10·8 %; I = 37·9 %; N = 4·2 %.

N-*Dimethylaminoacetic acid strontium chloride tetrahydrate.* On concentration of an aqueous solution prepared in the usual way, this compound crystallised as a network of needles (2·6 g.).

0·1943 g. dried at 120° lost 0·0425 g. H_2O = 21·9 %.
0·1943 g.; 0·1606 g. AgCl. Cl = 20·5 %.
0·2023 g.; NH_3 = 6·18 cc. *N*/10 acid. N = 4·3 %.
Calc. for $C_4H_9O_2N.SrCl_2.4H_2O$; H_2O = 21·6 %; Cl = 21·2 %; N = 4·2 %.

The mother-liquors gave a second crop on concentration.

N-*Dimethylaminoacetic acid strontium bromide tetrahydrate.* Under the foregoing conditions, long rods (2·9 g.) were obtained.

0·2035 g. dried at 120° lost 0·0335 g. H_2O = 16·5 %.
0·2035 g.; 0·1784 g. AgBr. Br = 37·3 %.
0·1951 g.; NH_3 = 5·24 cc. *N*/10 acid. N = 3·8 %.
Calc. for $C_4H_9O_2N.SrBr_2.4H_2O$; H_2O = 17·0 %; Br = 37·8 %; N = 3·3 %.

A further crop was obtained on concentration.

Di-(N-*dimethylaminoacetic acid*) *strontium iodide trihydrate.* On concentration of a solution obtained in the usual way, a first crop (0·8 g.) was obtained slightly impure. (Found: H_2O, 9·2; I, 37·6; N, 4·8 %; whence I : N = 1 : 1·16.) The second crop gave more correct figures:

0·1977 g. dried at 120° lost 0·0179 g. H_2O = 9·1 %.
0·1977 g.; 0·1518 g. AgI. I = 41·5 %.
0·1792 g.; NH_3 = 6·07 cc. *N*/10 acid. N = 4·7 %.
Calc. for $(C_4H_9O_2N)_2.SrI_2.3H_2O$; H_2O = 9·0 %; I = 42·2 %; N = 4·7 %.

N-Dimethylaminoacetic acid lithium chloride dihydrate. *N*-Dimethylaminoacetic acid hydrochloride (4·8 g.) was boiled with lithium carbonate (1 mol.); concentration gave bold tablets (3·3 g.).

0·1973 g. dried at 120° lost 0·0388 g. $H_2O = 19·7$ %.
0·1973 g.; 0·1547 g. AgCl. Cl = 19·7 %.
0·2002 g.; $NH_3 = 11·12$ cc. *N*/10 acid. N = 7·8 %.
Calc. for $C_4H_9O_2N.LiCl.2H_2O$; $H_2O = 19·9$ %; Cl = 19·5 %; N = 7·7 %.

N-Dimethylaminoacetic acid lithium bromide monohydrate and *di-*(*N-dimethylaminoacetic acid*) *lithium bromide.* When an equimolecular solution of *N*-dimethylaminoacetic acid and lithium bromide was concentrated, long needles (1·3 g.) were obtained, which corresponded approximately to the compound $C_4H_9O_2N.LiBr.1\frac{1}{2}H_2O$ (Found: H_2O, 11·5; Br, 35·2; N, 6·7 %; whence Br : N = 0·92 : 1); whilst on further concentration a crop of tablets (0·25 g.) of quite distinct appearance separated, approximating to the formula $(C_4H_9O_2N)_2.LiBr$. (Found: Br, 28·6; N, 9·1 %; whence Br : N = 1·1 : 2.)

To isolate each of these two compounds in a pure condition, separate solutions containing lithium bromide and *N*-dimethylaminoacetic acid in the proportion of 2 : 1 and 1 : 2 were concentrated. The solution containing excess lithium bromide deposited rectangular plates (2·0 g.) which were deliquescent.

0·2078 g. dried at 120° lost 0·0212 g. $H_2O = 10·2$ %.
0·2078 g.; 0·1846 g. AgBr. Br = 37·8 %.
0·1970 g.; $NH_3 = 9·79$ cc. *N*/10 acid. N = 7·0 %.
Calc. for $C_4H_9O_2N.LiBr.H_2O$; $H_2O = 8·7$ %; Br = 38·4 %; N = 6·7 %.

The second solution, containing two molecular proportions of *N*-dimethylaminoacetic acid, deposited hexagonal tablets (0·3 g.).

0·1212 g.; 0·0794 g. AgBr. Br = 27·9 %.
0·1457 g.; $NH_3 = 9·80$ cc. *N*/10 acid. N = 9·4 %.
Calc. for $(C_4H_9O_2N)_2.LiBr$; Br = 27·3 %; N = 9·5 %.

N-Dimethylaminoacetic acid lithium iodide hexahydrate. An equimolecular solution of lithium iodide and *N*-dimethylaminoacetic acid on concentration either on the water-bath or at room temperature, deposited mixed crystals. (Found in different preparations = I : N = 1 : 1·2.)

On this basis a third solution containing lithium iodide and *N*-dimethylaminoacetic acid in the proportion of 2 : 1 was concentrated at room temperature and gave rods (1·0 g.).

0·2112 g.; 0·1411 g. AgI. I = 36·1 %.
0·2060 g.; $NH_3 = 6·02$ cc. *N*/10 acid. N = 4·1 %.
Calc. for $C_4H_9O_2N.LiI.6H_2O$; I = 36·8 %; N = 4·1 %.

Di-(*N-dimethylaminoacetic acid*) *sodium chloride dihydrate.* After several unsuccessful attempts with 1 : 1 solutions, this compound was obtained crystallising in irregular octahedra from a solution containing two molecules of *N*-dimethylaminoacetic acid to one of sodium chloride by evaporation in a desiccator over sulphuric acid. It gradually decomposes when dried at 120°.

0·1981 g.; 0·0995 g. AgCl. Cl = 12·4 %.
0·1946 g.; $NH_3 = 12·67$ cc. *N*/10 acid. N = 9·1 %.
Calc. for $(C_4H_9O_2N)_2.NaCl.2H_2O$; Cl = 11·8 %; N = 9·3 %.

N-*Dimethylaminoacetic acid sodium bromide monohydrate* and *di*-(N-*dimethylaminoacetic acid*) *sodium bromide sesquihydrate.* Concentration of an equimolecular solution gave a mixture of long needles and hexagonal tablets; this mixture was re-dissolved and diluted, and on cooling tablets alone were obtained. (Found: Br, 30·2; N, 6·7 %; whence Br : N = 1 : 1·27.) On one recrystallisation the plates obtained gave Br, 25·8; N, 7·9 %; whence Br : N = 1 : 1·75, and after a further recrystallisation the pure sesquihydrate crystallising in rectangular tablets, which decompose on heating to 130°, was obtained.

0·1713 g.; 0·0969 g. AgBr. Br = 24·1 %.
0·1904 g.; NH_3 = 11·07 cc. *N*/10 acid. N = 8·1 %.
Calc. for $(C_4H_9O_2N)_2.NaBr.1\frac{1}{2}H_2O$; Br = 23·8 %; N = 8·3 %.

For the isolation of the pure 1 : 1 compound indicated by the first analysis, a solution containing two molecules of sodium bromide to one of *N*-dimethylaminoacetic acid was concentrated; after removal of a small amount of sodium bromide, the required compound separated as a monohydrate in hexagonal tablets which decompose slightly on drying at 120°.

0·2108 g.; 0·1780 g. AgBr. Br = 35·9 %.
0·1958 g.; NH_3 = 8·52 cc. *N*/10 acid. N = 6·1 %.
Calc. for $C_4H_9O_2N.NaBr.H_2O$; Br = 35·7 %; N = 6·3 %.

N-*Dimethylaminoacetic acid sodium iodide trihydrate.* This compound crystallises in rectangular plates.

0·2095 g.; 0·1582 g. AgI. I = 40·8 %.
0·2063 g.; NH_3 = 6·48 cc. *N*/10 acid. N = 4·4 %.
Calc. for $C_4H_9O_2N.NaI.3H_2O$; I = 41·3 %; N = 4·6 %.

N-*Dimethylaminoacetic acid potassium iodide sesquihydrate.* The first crop obtained (rectangular plates) was impure. (Found: I, 38·8; N, 5·4 %; whence I : N = 1 : 1·26); but a second crop of rectangular plates proved to be the pure compound.

0·1894 g.; 0·1504 g. AgI. I = 42·9 %.
0·2070 g.; NH_3 = 7·03 cc. *N*/10 acid. N = 4·8 %.
Calc. for $C_4H_9O_2N.KI.1\frac{1}{2}H_2O$; I = 42·9 %; N = 4·7 %.

Attempts to prepare addition compounds of potassium chloride or bromide were unsuccessful, the halide being recovered practically pure in each case.

Addition compounds of N-*trimethylaminoacetic acid* (*betaine*).

Penta-betaine di-calcium iodide undecahydrate. On concentration of a solution containing two molecules of betaine to one of calcium iodide, large, glistening, square plates were deposited which were optically isotropic. (Found: N, 5·2; I, 37·0 %; whence N : I = 1 : 0·79.) Recrystallisation gave similar plates. (Found: N, 5·2; I, 37·6 %; whence N : I = 1 : 0·8.)

When an equimolecular solution was concentrated, large, square plates were again obtained—with a similar ratio. (Found: N, 5·1; I, 37·5 %; whence N : I = 1 : 0·8), and on recrystallisation the same compound was obtained.

0·1843 g. dried at 95° lost 0·0169 g. H_2O = 9·2 %.
0·1566 g.; 0·1081 g. AgI. I = 37·3 %.
0·1835 g.; NH_3 = 6·91 cc. *N*/10 acid. N = 5·3 %.
0·1843 g.; C_2O_4Ca = 5·48 cc. *N*/10 $KMnO_4$. Ca = 6·0 %.
Calc. for $(C_5H_{11}O_2N)_5.(CaI_2)_2.11H_2O$; $7H_2O$ = 9·2 %; I = 37·0 %; N = 5·1 %; Ca = 5·8 %.

Attempts to prepare compounds of calcium chloride or calcium bromide were unsuccessful, only syrupy solutions being obtained.

Betaine barium iodide tetrahydrate. Concentration of a solution containing two molecules of betaine to one of barium iodide gave woolly needles (0·3 g.) which were impure. (Found: N, 4·8; I, 32·2 %; whence N : I = 1 : 0·75.) An equimolecular solution deposited a pure compound in rods (5·5 g.).

0·1959 g.; 0·1606 g. AgI. I = 44·3 %.
0·2080 g.; NH_3 = 3·36 cc. *N*/10 acid. N = 2·3 %.
Calc. for $C_5H_{11}O_2N.BaI_2.4H_2O$; I = 43·7 %; N = 2·4 %.

Betaine strontium chloride tetrahydrate. A solution of the components deposited long, silky needles (1·1 g.) on concentration.

0·1931 g. dried at 120° lost 0·0412 g. H_2O = 21·3 %.
0·1931 g.; 0·1558 g. AgCl. Cl = 20·0 %.
0·2100 g.; NH_3 = 6·11 cc. *N*/10 acid. N = 4·1 %.
Calc. for $C_5H_{11}O_2N.SrCl_2.4H_2O$; H_2O = 20·7 %; Cl = 20·4 %; N = 4·0 %.

Betaine strontium bromide pentahydrate. Concentration of a solution prepared in the usual way gave needles (1·0 g.) which were impure. (Found: N, 4·0; Br, 32·8 %; whence N : Br = 1 : 1·4.) The mother-liquor on concentration, however, gave a crop of needles (0·3 g.) which were analytically pure.

0·0984 g. dried at 120° lost 0·0199 g. H_2O = 20·2 %.
0·0984 g.; 0·0824 g. AgBr. Br = 35·6 %.
0·0908 g.; NH_3 = 1·99 cc. *N*/10 acid. N = 3·1 %.
Calc. for $C_5H_{11}O_2N.SrBr_2.5H_2O$; H_2O = 19·8 %; Br = 35·2 %; N = 3·1 %.

Di-betaine strontium iodide tetrahydrate. This compound was obtained in the usual way, crystallising in short needles (0·3 g.).

0·1390 g.; 1015 g. AgI. I = 39·4 %.
0·0722 g.; NH_3 = 1·99 cc. *N*/10 acid. N = 3·9 %.
Calc. for $(C_5H_{11}O_2N)_2.SrI_2.4H_2O$; I = 39·2 %; N = 4·3 %.

Betaine lithium bromide hydrate. On concentration of the solution, prepared in the usual manner, rods (2·4 g.) were deposited: they were impure. (Found: N, 5·6; Br, 24·6 %; whence N : Br = 1 : 0·77.) Recrystallisation gave long needles (0·5 g.) which were pure and contained $2\frac{1}{2}$ molecules of water.

0·1910 g. dried at 120° lost 0·0348 g. H_2O = 18·2 %.
0·1910 g.; 0·1431 g. AgBr. Br = 31·9 %.
0·1918 g.; NH_3 = 7·52 cc. *N*/10 acid. N = 5·5 %.
Calc. for $C_5H_{11}O_2N.LiBr.2\frac{1}{2}H_2O$; H_2O = 18·1 %; Br = 32·1 %; N = 5·6 %.

Di-betaine lithium iodide monohydrate. Tufts of needles (1·0 g.) were obtained on concentration of an equimolecular solution.

0·1966 g.; 0·1198 g. AgI. I = 32·9 %.
0·2094 g.; NH_3 = 10·28 cc. *N*/10 acid. N = 6·9 %.
Calc. for $(C_5H_{11}O_2N)_2.LiI.H_2O$; I = 32·9 %; N = 7·2 %.

Lithium chloride. When an equimolecular solution of lithium chloride and betaine was concentrated to a syrup, a mixture of needles and small hexagonal

plates was slowly deposited. But when two solutions, one with the components in the ratio 2 : 1 and the other in the ratio 1 : 2, were concentrated, no crystalline substances could be isolated.

Betaine sodium chloride sesquihydrate. On concentration of an equimolecular solution a mixture was obtained. A second solution, however, containing twice the amount of betaine, when concentrated, gave long needles alone.

0·1918 g. dried at 120° lost 0·0294 g. $H_2O = 15·3$ %.
0·1918 g.; 0·1375 g. AgCl. Cl = 17·7 %.
0·2040 g.; $NH_3 = 10·23$ cc. *N*/10 acid. N = 7·0 %.
Calc. for $C_5H_{11}O_2N.NaCl.1\frac{1}{2}H_2O$; $H_2O = 13·3$ %; Cl = 17·5 %; N = 6·9 %.

Di-betaine sodium bromide sesquihydrate. The first crop obtained when an equimolecular solution was concentrated consisted of rods (1·1 g.) which were impure. (Found: N, 4·6; Br, 35·6 %; whence N : Br = 1 : 1·36.) Concentration of the mother-liquor gave a second crop of glistening, rectangular leaflets (0·3 g.) which analysed correctly.

0·1078 g. dried at 120° lost 0·0093 g. $H_2O = 8·6$ %.
0·1078 g.; 0·0554 g. AgBr. Br = 21·9 %.
0·1359 g.; $NH_3 = 7·23$ cc. *N*/10 acid. N = 7·5 %.
Calc. for $(C_5H_{11}O_2N)_2.NaBr.1\frac{1}{2}H_2O$; $H_2O = 7·4$ %; Br = 21·9 %; N = 7·7 %.

Betaine sodium iodide trihydrate. Rectangular plates (0·4 g.) were deposited on concentrating an equimolecular solution. They were slightly impure. (Found: N, 3·9; I, 38·1 %; whence N : I = 1 : 1·08.) The second crop, also rectangular plates, proved to be pure.

0·1985 g.; 0·1449 g. AgI. I = 39·4 %.
0·2001 g.; $NH_3 = 5·89$ cc. *N*/10 acid. N = 4·1 %.
Calc. for $C_5H_{11}O_2N.NaI.3H_2O$; I = 39·2 %; N = 4·4 %.

Betaine ammonium iodide dihydrate. An equimolecular solution deposited rods on concentration. (Found: N, 9·1; I, 45·1 %; whence N : I = 1 : 0·54.)

The rods were recrystallised, and after removing a small quantity of leaflets, rods were again deposited.

0·2007 g. dried at 120° lost 0·0129 g. $H_2O = 6·4$ %.
0·2007 g.; 0·1575 g. AgI. I = 42·4 %.
0·2024 g.; $NH_3 = 13·24$ cc. *N*/10 acid. N = 9·2 %.
Calc. for $C_5H_{11}O_2N.NH_4I.2H_2O$; $H_2O = 6·0$ %; I = 42·6 %; N = 9·4 %.

From solutions of betaine and ammonium chloride, ammonium bromide and potassium chloride, no addition compounds could be obtained.

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CLVII. THE SECOND PROTEIN (LIVETIN) OF EGG-YOLK.

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(*Received August 31st, 1928.*)

If the usual method is adopted for the preparation of lecitho-vitellin from hen's egg-yolk, namely, dilution of the egg-yolk with an equal volume of 10 % sodium chloride solution, thorough extraction of the mixture with ether, followed by dilution of the resulting clear aqueous layer with 15 to 20 volumes of water, it will be found that whilst the lecitho-vitellin is almost quantitatively precipitated, there is still an appreciable amount of protein-nitrogen in the filtrate [Gross, 1899, quoted by Plimmer]. Plimmer [1908] by acidifying the filtrate with acetic acid and boiling to coagulate the protein, obtained several grams of this material, which he called "livetin." He determined the nitrogen partition by the Hausmann method, and found that the figures agreed fairly closely with those obtained for vitellin, but differed from those obtained for ovalbumin. Since this work, little seems to have been done on this second protein of egg-yolk. Apart from the careful analyses of Plimmer just mentioned, we are not aware of any information as to its composition, or even as to the class of protein to which it belongs. Moreover Plimmer, although he brought evidence to show that his product was not ovalbumin, did not demonstrate clearly that the proteins of the white did not contribute to his coagulum. He put forward the suggestion that the product he separated might be closely related to vitellin—in fact, vitellin minus the phosphorus-containing fraction of the molecule.

During the course of the preparation of some pure ovovitellin we have taken the opportunity of examining our residues for this little studied substance, and are now able to present some more definite data as to its nature and properties.

Preparation of livetin.

We find that it is difficult, if not impossible with some eggs, to free the yolk quantitatively from adherent white by washing it simply in running water as did Plimmer. To be quite sure that the yolk is clean, we find it necessary to wash it in three or four changes of 0·9 % sodium chloride (to dissolve the globulin satisfactorily), and also to remove the chalazae with scissors. The yolk is further cleaned by allowing it to roll slowly down a strip of dry

calico sheeting, returning it, if necessary, to be washed again in the salt solution. It is finally dried, as far as possible, on the sheeting. In this way the white can be completely removed. Lecitho-vitellin is prepared from the clean yolks in the usual way[1], leaving invariably a considerable quantity of non-vitellin protein-nitrogen in the final filtrate. By half saturation with ammonium sulphate almost the whole of this protein may be precipitated. The filtrate from this precipitate gives only a very slight opalescence with trichloroacetic acid, thus demonstrating the absence of appreciable amounts of albumin. The precipitate is taken up with water, the concentration of ammonium sulphate brought up to 25 %, and the slight precipitate filtered off. To the clear filtrate sufficient saturated ammonium sulphate solution is added to bring the concentration of the salt to half saturation, the precipitated livetin filtered off, redissolved, and again precipitated. Solution and precipitation are repeated once more, the precipitated protein is then dissolved in the minimal quantity of water and the lipins are removed by alcohol-ether extraction at $-15°$ [Hewitt, 1927]. The extracted protein is freed from alcohol and ether by Hewitt's method, only a small fraction undergoing denaturation during the process. The dried, extracted protein, containing ammonium sulphate, is taken up in water, filtered, reprecipitated with ammonium sulphate, dissolved in the minimal quantity of water, and the solution, adjusted to p_H 5·0, is dialysed in closed collodion sacs at 3° against repeated changes of distilled water until at least two days after the dialysate has ceased to give the faintest opalescence with barium chloride.

Although there are now only traces of salts in the sacs, the livetin is still in solution. Its behaviour is therefore that of a pseudoglobulin. In a few cases the dialysed solutions are opalescent, and may be clarified by filtration through paper pulp. On adding the clear, dialysed solution to 10 volumes of distilled water, an opalescence is produced, which disappears on the addition of dilute sodium chloride or ammonium sulphate solution. On continuing the dialysis for some weeks, a portion of the protein is precipitated.

Examination of livetin.

Qualitative and precipitation tests.

A 0·6 % solution of livetin gives a precipitate on boiling alone, or on boiling with very dilute acetic acid, a precipitate in the cold with three volumes of alcohol or acetone, with trichloroacetic, picric, tungstic and sulphosalicylic acids, with the salts of the heavy metals, and with colloidal ferric hydroxide. It is completely precipitated by half saturation with ammonium sulphate, by saturation with magnesium sulphate or sodium chloride (after 24 hours'

[1] A short dialysis of the lecitho-vitellin-NaCl solution against distilled water before precipitation of the phosphoprotein with water considerably reduces the amount of dilution necessary for its complete separation, and hence diminishes the bulk of the filtrate from which the livetin has to be precipitated.

standing in the case of the last two salts). It gives the biuret, xanthoproteic and Millon's reactions, a strong glyoxylic reaction, but a very weak, almost negative, Molisch reaction. With a 4 % solution of the protein, a positive sulphur reaction is given with sodium hydroxide and lead acetate. After hydrolysis with trypsin it gives a strong Pauly's reaction, and a definite but weak Totani's "secondary reaction" for histidine.

Quantitative analysis.

1. *Nitrogen, phosphorus and sulphur.* Livetin, prepared by adding to its solution in dilute ammonium sulphate, at the stage before alcohol-ether extraction, three volumes of alcohol and heating to boiling, gave, after washing the coagulated protein with water and alcohol, and drying at 100°, ash = 1 %; N = 15·12 %; P = 0·12 %. (Plimmer found N varying between 14·8 % and 15·2 %, and P values between 0·1 % and 0·65 % in five different specimens of livetin.)

A purer specimen after alcohol-ether extraction gave ash = 0·6 %; N = 15·35 %; P = 0·067 %, *i.e.* a much lower content of phosphorus. On our two purest specimens of livetin the N : P ratio was 1 g. N : 3·13 mg. P, and 1 g. N : 3·15 mg. P, respectively. This phosphorus is not extractable by alcohol-ether, either in the cold or on boiling. It is rather less than one-half of the amount of P per gram of N present in extracted egg-albumin [Hewitt, 1927]. Assuming that there is only one atom of P in each molecule of protein, the minimum molecular weight of livetin works out at about 64,000.

The total sulphur content of livetin is 1·80 % (Carius); this high figure agrees with the finding of a relatively large quantity of cystine in the molecule (see next paragraph). Taken in conjunction with the results shortly to be described for the ratio lecitho-vitellin : livetin in the yolk, and for the sulphur content of the former protein, it appears that about one-half of the total sulphur of the yolk is originally present as livetin.

2. *Amino-acids.* Using the method of Folin and Ciocalteu [1927] for tyrosine and tryptophan and that of Folin and Looney [1922] for cystine, the following values have been obtained for the percentage of these amino-acids in purified livetin (three specimens):

tyrosine 5·14, 5·20, 5·17; mean 5·17;
tryptophan 2·08, 2·34, 2·00; mean 2·14;
cystine 3·5, 4·3, 3·9[1]; mean 3·9 (= 1·05 % cystine-sulphur).

According to analyses made at the same time, vitellin contains 5·0 % tyrosine, 1·6 % tryptophan and only 1·4 % cystine, with a total sulphur content of 0·6 %. Lecitho-vitellin contains only 0·5 % sulphur. Plimmer's suggestion of the possibility of a direct relationship between vitellin and livetin is not borne out by these results.

[1] Specimen of livetin from yolk of duck's egg.

Physical constants.

A preliminary investigation of some of the physical constants of the pure, dialysed protein has been made.

1. *The c_H for minimum solubility.* This was determined approximately by adding small portions of the protein solutions to acetate buffers, and then adding neutral alcohol till precipitation occurred. Using 2 cc. buffer, 0·3 cc. of 3·2 % livetin solution and 1 cc. alcohol, maximum precipitation occurred between p_H 4·8 and 5·2. A second experiment with the same livetin solution gave optimal precipitation at p_H 5·0. A different specimen of the protein (2·1 % solution) gave minimum solubility at p_H 4·8[1]. It may therefore be concluded that the isoelectric point of livetin is in the neighbourhood of p_H 4·8–5·0. The titration curve of the purified protein, kindly determined by Dr J. R. Marrack, indicates a broad isoelectric zone in this region.

2. *The refractive index* of the aqueous solution was determined using Pulfrich's dipping refractometer and a glass mercury-vapour lamp. At 20°, four alcohol-ether-extracted specimens gave 0·00184; 0·00192; 0·00189; 0·00196; mean 0·00190, as the refractive index for 1 % protein. Pure ovalbumin in the same apparatus gave a mean value for 1 % protein of 0·00192.

3. *The optical rotation* of several specimens of the protein, prepared by slightly different methods, and in solutions of different concentrations was kindly determined for us by Dr L. F. Hewitt. The specific rotation varied considerably with the purity of the material; for pure, alcohol-ether-extracted specimens the following results were obtained:

$[\alpha]^{20}_{5461}$ = (to nearest degree) − 55°; − 53°; − 56°; − 58°; mean − 55·5°.

The pure protein appears, therefore, to have a slightly higher specific rotation than ovalbumin, and a rather lower one than serum-globulin [cf. Hewitt, 1927, Table V].

QUANTITY OF LIVETIN PRESENT IN THE YOLK.

A method for the estimation of the amount of livetin in a single yolk has been devised. The yolk is cleaned and dried as previously described, then transferred to a 50 cc. cylinder, the vitelline membrane being removed with forceps. (If absolute values are required, it is necessary to introduce obvious modifications here.) The volume is measured, an equal volume of 8 % NaCl is added, and the whole stirred vigorously till the mixture is homogeneous. 20 cc. of the diluted egg-yolk is transferred to a graduated, stoppered cylinder, and shaken with 40 cc. ether containing 2·5 % alcohol. The ether is removed after the layers have separated, and the extraction repeated thrice with 20 to 25 cc. of ether. The ether is removed as completely as possible and 4 % NaCl

[1] It is of interest to record that similar concentrations of pure ovalbumin, in these buffer solutions, required only one-fifth of this amount of alcohol to produce a turbidity; *i.e.* ovalbumin, though less readily precipitated by salt solutions, is much more sensitive to the presence of alcohol than is livetin.

solution added to make the volume up to 25 cc. 0·5 cc. is removed in duplicate for total N determination (micro-Kjeldahl) (*A*). 20 cc. are diluted to 200 cc. with distilled water. After standing overnight to allow the precipitated lecitho-vitellin to settle, the supernatant liquid is filtered. The protein in this filtrate is almost exclusively livetin. 20 cc. are taken in duplicate for total N determination (*B*). 80 cc. are treated with 20 cc. 25 % trichloroacetic acid, and filtered after standing for 15 minutes; total N is determined in duplicate on 40 cc. of the filtrate (*C*). The livetin in the original yolk may readily be calculated from the difference between *B* and *C*, whilst the difference between *A* and *B* gives the lecitho-vitellin content. (Livetin is assumed to contain 15 % and lecitho-vitellin 13 % N.)

By this method, the following values have been found:

Egg	Ratio lecitho-vitellin : livetin
Hen	3·83 : 1, 3·46 : 1, 3·89 : 1, 3·56 : 1, 3·62 : 1, 3·40 : 1; mean 3·63 : 1
Duck	3·96 : 1, 3·99 : 1, 3·35 : 1; mean 3·76 : 1

With the duck's egg the separation of the ether extract in the first stages of the determination is distinctly more difficult than with the hen's egg. A trace of capryl alcohol and the device of keeping the long extraction cylinder horizontal assist the separation.

It will be observed that, making allowance for certain almost inevitable experimental errors, the ratio lecitho-vitellin : livetin appears to be fairly constant from one egg to another, and possibly from one species to another. In the values given above for hens' eggs, the first four figures were from commercial "new-laid" eggs selected at random, the last two figures were from eggs 24 hours old, of pedigree Rhode Island Red parentage.

Between one-quarter and one-fifth of the protein of the yolk of the egg, therefore, is livetin, the absolute amount being, for an egg of average size, 0·6 to 0·9 g.

Is livetin a single protein?

Throughout this paper it has been tacitly assumed that the livetin of egg-yolk, prepared in the manner described, is a single individual. The method given above for determining the livetin in a single egg-yolk presupposes, in fact, that all the non-vitellin protein in egg-yolk is the same substance—livetin.

As against this assumption, it must be pointed out that (*a*) there is a small amount of protein precipitated by 25 % saturation with ammonium sulphate; (*b*) although there is only a trace of protein in the filtrate from 50 % ammonium sulphate precipitation, there appears to be no very sharp zone of precipitation between 25 and 50 % saturation in which the rest of the protein comes down quantitatively; (*c*) on long continued dialysis it appears possible to separate the livetin into two fractions, one more soluble in water than the other. So far the fractions have not been examined separately, but further work on this point is in hand.

In view of the work of Sørensen [1925] and of Linderström-Lang [1925], who have separated serum-euglobulin and pseudoglobulin and caseinogen into

fractions having different solubilities and phosphorus contents, the whole of our criteria as to the individuality of proteins may have to be revised. For the present it is justifiable to say that the preliminary examination of the non-vitellin protein of egg-yolk has revealed that by far the greater portion of it (90 % or more) behaves, by the usual criteria adopted up to now for protein characterisation, as a single individual.

SUMMARY.

1. It is confirmed that another protein beside vitellin is present in the yolk of the hen's or the duck's egg. This protein has been isolated in a fairly pure, undenatured form for the first time.

2. This substance, for which the name livetin suggested by Plimmer is retained, has, on the whole, the properties of a pseudoglobulin. It contains 5·2 % tyrosine, 2·1 % tryptophan, 3·9 % cystine, 0·05 % phosphorus, and 1·8 % sulphur. It has its isoelectric point in the neighbourhood of p_H 4·8–5·0, refractive index in 1 % aqueous solution = 0·00190 at 20°, $[\alpha]^{20}_{5461} = -55{\cdot}5°$. It is probably unrelated to ovalbumin or vitellin. Its high content of the indispensable amino-acids cystine and tryptophan suggests that it may have a considerable biological value.

3. A method is described for estimating the amount of this protein in a single egg-yolk. In the fresh yolk, the ratio vitellin : livetin is fairly constant from one egg to another. Between one-quarter and one-fifth of the yolk-proteins of the hen's or duck's egg is livetin.

This work has been carried out with the aid of grants from the Research Fund of the London Hospital Medical College.

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CLVIII. CONSTITUTION OF HEXOSE-DIPHOSPHORIC ACID. PART II.

THE DEPHOSPHORYLATED α- AND β-METHYLHEXOSIDES[1].

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(*Received September 7th, 1928.*)

The researches of Harden and Young [1909, 1910] showed that the three monosaccharides glucose, mannose and fructose behave in the same way towards yeast juice in the presence of added phosphate. The identity of the hexosediphosphoric acid produced in each case was established by Young [1909], who showed that this ester on acid hydrolysis yielded free phosphoric acid and a laevo-rotatory solution from which fructose could be isolated. The rotation of this solution, however, was always lower than that of a fructose solution of equal reducing power, and this caused Young to avoid drawing definite conclusions as to the nature of the sugar in hexosediphosphoric acid. Later Neuberg and Kretschmer [1911] succeeded in isolating crystalline fructose from the products of hydrolysis and they concluded that this was the only sugar present and that the original acid was fructosediphosphoric acid.

At this time the reactive γ-sugars had not been described, for it was not until some years later that Fischer [1914] prepared γ-methylglucoside, the first representative of the structural isomerides containing an unstable oxide ring. The recognition that inulin and sucrose are derivatives of γ-fructose followed from the work of Haworth [1920] and Irvine and Steele [1920] and in 1922 Menzies [1922] isolated γ-methylfructoside from the products of esterification of ordinary fructose by Fischer's method.

In the light of this more recent knowledge of structural isomerism in the sugar group the further investigation of the constitution of hexosediphosphoric acid was undertaken in order to determine the type of oxide ring in the hexose molecule as well as the position of the phosphoric acid groups. Of these two groups the position of one followed with considerable certainty from the work of Young [1911] on the reaction of hexosediphosphoric acid with phenylhydrazine. Using cold reagents he obtained the phenylhydrazine salt of a

[1] The conclusions reached in this paper were communicated to the Biochemical Society on December 9th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, **46**, 1183.

phenylhydrazone in which both phosphoric acid groups were still present. On heating, the osazone was formed, one phosphoric acid group however being simultaneously eliminated. From these facts Young concluded that if the parent hexose is fructose one phosphoric acid group is attached to the terminal carbon atom adjacent to the carbonyl group. No evidence was forthcoming as to the position of the second group.

The preparation of methylhexosidediphosphoric acid and the separation of the α- and β-isomerides by crystallisation of their brucine salts has already been described [Morgan, 1927] and in the present paper experimental proof is given that the methylhexosidediphosphoric acids are derivatives of γ-fructose and the bearing of this conclusion on the constitution of the free phosphoric ester is discussed.

The removal of the phosphoric acid groups from the α- and β-methylhexoside-diphosphoric acids by the bone phosphatase.

By the action of the bone phosphatase it has been possible to eliminate the phosphoric acid groups from both the α- and β-methylhexosidediphosphoric acids without removing the glucosidic methyl group, thus confirming and extending the preliminary experiment of Morgan [1927]. For most of the experiments here described we have used active preparations of the bone phosphatase purified by precipitation from the aqueous extract of bones of young rabbits by a mixture of ether and alcohol [Martland and Robison, 1928] and have in this way avoided contamination of the reaction products with alcohol-soluble material derived from the bones.

The barium salts of the methylhexosidediphosphoric acids were dissolved in water and treated with the bone phosphatase preparation. The flasks containing the mixture were immersed in a water-bath at 38° and the p_H of the solution during the hydrolysis was maintained at 8·6–8·8 by the addition of cold saturated baryta at short intervals. Under these conditions the inorganic phosphate liberated by the action of the enzyme was at once precipitated as barium phosphate. The progress of the hydrolysis was roughly indicated by the amount of baryta added but in practice this amount was usually considerably less than the equivalent of the inorganic phosphate set free, probably owing to the precipitation of a portion of the latter as $BaHPO_4$ in place of $Ba_3(PO_4)_2$. For example, 0·6 g. bone enzyme acting on 4 g. barium methylhexosidediphosphate set free about 95 % of the phosphoric acid in 12 hours while the amount of baryta added corresponded with no more than 70 %. At the close of the hydrolysis the enzyme and any barium salts remaining in solution were precipitated by the addition of three volumes of alcohol and the alcoholic filtrate was evaporated to dryness *in vacuo*. The syrupy residue was dissolved in absolute alcohol and the filtered solution again evaporated to dryness. A blank experiment was in all cases carried out with the enzyme preparation alone but the amount of the alcohol-soluble matter so obtained did not exceed 2 % of the original weight of the enzyme pre-

paration. The small residues obtained in these blank experiments were optically inactive and possessed only very slight reducing properties either before or after acid hydrolysis.

The results of a number of experiments carried out on various specimens of α- and β- and the mixed $\alpha\beta$-methylhexosidediphosphates are shown in Table I.

Table I.

Barium methylhexoside-diphosphate		Hydrolysed by enzyme	Dephosphorylated methylhexoside: Reducing power mg. glucose	Reducing power after acid hydrolysis mg. glucose	Equiv. weight of hexoside mg.	MeO in dried hexoside syrup %	$[\alpha]_{5461}$ of hexoside	$[\alpha]_{5461}$ of hexose formed by acid hydrolysis
Isomer	$[\alpha]_{5461}$	%						
α	+ 8·3°	—	4·0	83	89	—	+64°	−103°
α	+ 8·9°	98	0·0	233	251	—	+54°	—
α	+ 8·3°	—	7·0	298	321	—	+48°	—
β	−10·4°	—	0	280	302	14·7	−51°	−109°
β	− 8·3°	98	0	274	295	—	−44°	—
β	− 9·7°	—	2·0	65	70	—	−47°	−101°
β	− 9·0°	—	1·2	85	92	—	−44°	—
$\alpha\beta$	—	91	3·6	782	848	15·2	—	−109°
$\alpha\beta$	—	96	8·4	846	910	15·6	—	—
Methyl fructoside requires			—	—	—	15·9	—	−110·8° (fructose)

A small portion of the syrupy hydrolysis product was dried to constant weight *in vacuo* at 78° over phosphorus pentoxide and determinations of the methoxy-group were made on this material by the micro-Zeisel method. The specific rotations were determined on aqueous solutions of the partially dried syrups, the weight of hexoside being calculated from the reducing power (Hagedorn and Jensen) after acid hydrolysis. A small error is introduced by this method of calculation owing to the slight destruction of sugar which occurs during the hydrolysis in spite of the rapidity with which the latter takes place. This is shown by the following figures obtained for the reducing power after heating the α- and β-methylhexosides with 0·1 *N* hydrochloric acid at 100° for varying periods.

Reducing power as mg. glucose.

	Time of heating			
	0 min.	3 min.	5 min.	10 min.
α-Methylhexoside	0	233	227	215
β-Methylhexoside	0	274	271	264

The small reducing power shown by some of the syrups before acid hydrolysis and due in part to material derived from the enzyme preparation has also been neglected in these calculations. In view of these possible errors and of the variations in the specific rotations of the original barium methylhexosidediphosphates (see Table I) the values for the specific rotation of the methylhexosides show fair agreement.

The sensitiveness of the methylhexosides to acids is more clearly seen in Table II which shows the progress of hydrolysis of the α- and β- and of the mixed $\alpha\beta$-isomers by 0·01 *N* acid at room temperature. The final values obtained for the specific rotation of the hexose thus formed are in satisfactory agreement with that of $\alpha\beta$-fructose (normal), $[\alpha]_{5461} = -110{\cdot}8°$. The extreme ease of hydrolysis and the specific rotation of the methylhexosides suggested, however, that the latter were not derivatives of the normal but of γ-fructose.

Table II. *Hydrolysis of methylhexosides with* 0·01 N *HCl.*

	α-Methylhexoside vol. 10 cc., temp. 18°		β-Methylhexoside vol. 10 cc., temp. 18°			Mixed αβ-hexoside vol. 15 cc., temp. 22–23°	
Time (hours)	α observed (l=1)	Reducing power as glucose mg.	α observed (l=1)	Reducing power as glucose mg.	Time (hours)	α observed (l=2)	Reducing power as glucose mg.
0	+0·19°	1·2	−0·13°	0·17	0	+0·05	0
4	+0·16°	—	—	—	4	+0·01	1·35
20	+0·12°	5·4	−0·16°	2·5	23	−0·08	6·8
27	+0·05°	6·3	−0·17°	3·1	49	−0·15	—
44	+0·02°	9·3	−0·18°	4·8	72	−0·19	12·3
92	−0·04°	14·8	−0·19°	8·5	96	−0·225	—
116	−0·09°	—	−0·19°	—	120	−0·245	—
140	−0·13°	—	−0·20°	—	168	−0·29	19·8
208	−0·19°	—	−0·215°	—	192	−0·29	19·8
280	−0·25°	26·4	−0·23°	17·5			
336	−0·29°	29·2	−0·24°	22·0			
	$[\alpha]_{5461}$ of hexose = −99°		$[\alpha]_{5461}$ of hexose = −109°			$[\alpha]_{5461}$ of hexose = −109°	

Irvine and Patterson [1922] and Menzies [1922] showed that β-methyl-fructoside when dissolved in 0·009 *N* hydrochloric acid at room temperature possessed a constant rotation for several days, while under parallel conditions the rotation of methyl-γ-fructoside changed from dextro ($[\alpha]_D + 22°$) to laevo in 30 hours and reached the value $[\alpha]_D - 64°$ in 27 days. The specific rotations of the various methylfructosides recorded in the literature so far as we have been able to ascertain are given in Table III. The figures in brackets give the values of $[\alpha]_{5461}$ calculated from those for $[\alpha]_D$ by means of the factor 1·18.

Table III.

	$[\alpha]_D$	$[\alpha]_{5461}$	Reference
α-Methylfructoside (normal)	+45°	(+53°)	Schlubach and Schröter [1928]
β-Methylfructoside (normal)	−172°	(−203°)	Hudson and Brauns [1916]
α-Methyl-γ-fructoside	+65 to +70°*	—	Schlubach and Rauchalles [1925]
β-Methyl-γ-fructoside	−17°*	—	Schlubach and Rauchalles [1925]
αβ-Methyl-γ-fructoside	+26°	(+31°)	Menzies [1922]
α-Methylhexoside from methyl-hexosidediphosphoric acid	—	+55° (mean)	Present communication
β-Methylhexoside from methyl-hexosidediphosphoric acid	—	−47° (mean)	Present communication

* These values were calculated from results of experiments on the hydrolysis of $\alpha\beta$-methyl-γ-fructoside by invertase and are not claimed by the authors as more than approximations to the correct figures.

Tetramethylmethylhexosides.

Further confirmation that the α- and β-methylhexosides possessed the γ or butylene oxide ring was obtained by converting them into the corresponding fully methylated tetramethyl-α- or -β-methylhexoside by the action of silver oxide and methyl iodide [Purdie and Irvine, 1903]. These methylated derivatives were then converted into the tetramethylhexose by removal of the glucosidic methyl group by hydrolysis with dilute acid.

The very small quantities of the original methylhexosides, the poor yields obtained in the methylation and the unstable nature of the products added considerably to the difficulty of obtaining the tetramethylhexose in sufficiently pure condition for identification. Five successive methylations were carried out, in the first two of which a small quantity of methyl alcohol was added as an extraneous solvent. In the subsequent methylations the material was soluble in methyl iodide.

We did not succeed in raising the percentage of methoxyl in the crude methylated product above 50 % against a theoretical value of 62 % for tetramethylmethylhexoside. It was, however, found possible to purify these syrups by fractional distillation under pressures of about 1 mm., and although, when applied to such small quantities, this method could not be expected to yield perfectly pure products, a considerable improvement was effected, as can be seen from the percentage of CH_3O in the distillate (Table IV).

Table IV.

Hexoside methylated	CH_3O before distillation %	CH_3O after distillation %	Fraction collected	Weight of distillate mg.
α	44·6	50·3	—	38
β	49·6	56·8	100–110° (1–1·5 mm.)	88
β	49·6	54·8	—	54
$\alpha\beta$	49·8	61·7	87–100° (0·5 mm)	180

Hydrolysis of the tetramethylmethylhexosides.

The fully methylated non-reducing hexosides were converted into the tetramethylhexose by hydrolysis with dilute mineral acid. The compounds were dissolved in water and concentrated hydrochloric acid was added to give a 0·1 *N* solution. The rotation became constant after heating for 3–5 minutes at 100°, showing that hydrolysis was complete. Table V gives the values of the specific rotations of the tetramethylmethylhexosides together

Table V.

Methylated product derived from	Before hydrolysis (tetramethyl-methylhexoside) $[\alpha]_{5461}$	After hydrolysis (tetramethylhexose) $[\alpha]_{5461}$
α-Hexoside	+15·5°	+25·6°
β-Hexoside	− 6·8°	+31·9°
$\alpha\beta$-Hexoside	− 1·3°	+40°

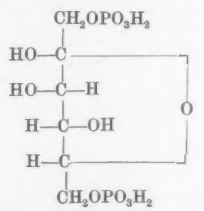
with those of the tetramethylhexose produced on hydrolysis. The three values for the tetramethylhexose should be identical since on removing the glucosidic methyl group the equilibrium mixture of α- and β-forms would be obtained; but the purity of the products probably varied with the amount of the tetramethylmethylhexoside distilled, this amount being least in the case of the α-compound and greatest in the case of the $\alpha\beta$-mixture. The tetramethylhexose obtained from the latter gave a value of 52·1 % CH_3O against 52·5 % calculated for tetramethylfructose. The specific rotation of this product ($[\alpha]_{5461} + 40°$) is in close agreement with that found by Irvine and Steele [1920] and Haworth [1920] for tetramethyl-γ-fructose ($[\alpha]_D + 31°$) but widely different from the specific rotation of crystalline (normal) tetramethylfructose ($[\alpha]_D - 121°$; [Purdie and Paul, 1907]). From these results we therefore conclude that the isomeric methylhexosides prepared from hexosediphosphoric acid were derivatives of γ-fructose.

Constitution of hexosediphosphoric acid.

The formation of methyl-γ-fructoside from hexosediphosphoric acid does not at once prove that the latter compound is itself a derivative of γ-fructose, since it has been shown by Purdie and Paul [1907] and Menzies [1922] that ordinary fructose, esterified by Fischer's method, yields a mixture of normal and γ-methyl derivatives. However, the $\alpha\beta$-methylhexoside which yielded the purest tetramethyl-γ-fructose ($[\alpha]_{5461} + 40°$) represented about 80 % of the theoretical yield from the original hexosediphosphoric acid and neither in this nor in other preparations was evidence obtained of other isomers of different properties which might correspond with the more stable normal derivatives.

This would seem to indicate that the free acid also possesses the γ-fructose structure but it is hoped that further work now in progress will yield more conclusive evidence on this point.

The presence of the butylene oxide ring in the methylhexosidediphosphates implies that neither of the phosphoric acid groups can be in position 5; while, if the presence of a similar oxide ring in the free hexosephosphoric acid is accepted, we may infer that one phosphoric acid group is in position 6 since if this terminal CH_2OH group were unsubstituted we should expect the stable amylene oxide ring to be formed. Accepting Young's conclusion as to the position of the other phosphoric acid group we suggest therefore that hexosediphosphoric acid is γ-fructose-1 : 6-diphosphoric acid.



If this structure proves to be correct hexosediphosphoric acid will fall into line with other naturally occurring fructose derivatives, sucrose, raffinose, inulin, in which the fructose has been shown to possess the unstable butylene oxide ring.

Summary.

1. By the action of the bone phosphatase on the α- and β-methylhexoside-diphosphoric acids the corresponding α- and β-methylhexosides have been obtained as non-reducing syrups.

2. The properties of these compounds agree with those expected for α- and β-methyl-γ-fructosides.

3. These methylhexosides have been further converted into fully methylated derivatives from which tetramethyl-γ-fructose has been obtained by acid hydrolysis.

4. The bearing of these facts on the constitution of hexosediphosphoric acid is discussed and it is suggested that this compound is γ-fructose-1 : 6-diphosphoric acid.

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CLIX. TREHALOSEMONOPHOSPHORIC ESTER ISOLATED FROM THE PRODUCTS OF FERMENTATION OF SUGARS WITH DRIED YEAST.

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(*Received September 12th, 1928.*)

Since the original work of Harden and Young on the function of phosphates in alcoholic fermentation, the idea that the formation of phosphoric esters may be an essential stage not only in the biochemical degradation of hexoses but also in the condensation of these sugars to the polysaccharides has taken firm hold of biochemical imagination. The experimental evidence, which consists in (*a*) the stimulating effect of phosphates on certain processes involving carbohydrates in living tissues or tissue extracts, (*b*) the occurrence of phosphoric esters among the reaction products, is admittedly insufficient to prove such a general conception, but this remains a stimulating basis for research. As to the manner in which the introduction of the phosphoric acid group furthers such reactions we have, as yet, very little knowledge.

Of the two phosphoric esters hitherto isolated from the products of alcoholic fermentation, hexosediphosphoric acid is probably the 1 : 6-diphosphoryl derivative of γ-fructose [Young, 1909, 1911; Morgan, 1927; Morgan and Robison, 1928], although the proof of this structure is not yet complete. The constitution of the hexosemonophosphoric acid is still unknown but certain facts set out in the account of its isolation and investigation [Robison, 1922] point to it being a mixture of monophosphoric esters of glucose and fructose. This view is supported by the evidence [Meyerhof and Lohmann, 1927] of its reducing power towards sodium hypoiodite, a reagent which distinguishes between aldoses and ketoses. It is possible that one of the components may prove to be identical with the monophosphoric ester obtained by Neuberg [1918] by the partial hydrolysis of hexosediphosphoric ester by acids and by the enzymes of *Aspergillus oryzae* and of yeast [Neuberg and Leibowitz, 1927, 1, 2].

During the isolation of hexosemonophosphoric acid from the products of the action of yeast juice on hexoses indications were obtained of the presence of other esters, in particular of a compound with properties intermediate between those of the mono- and di-phosphates, but the results of experiments

carried out at that time were not sufficiently conclusive to warrant publication. During the past two years this and other points of interest arising out of the early work have been reinvestigated, employing micro-methods of analysis which have made it possible to follow the progress of the fractionations at every stage. The full account of these experiments will be given in a later communication. The present paper is confined to the description of a new ester, entirely distinct from the compound referred to above, which has been isolated from the products of the fermentation of hexoses by dried yeast in presence of phosphates. This ester has proved to be a monophosphoric ester of the non-reducing disaccharide trehalose, a glucose glucoside found in trehala manna (excreted by the insect *Larinus maculatus*), in certain fungi, seaweeds and yeasts.

A series of fermentation experiments was carried out with various samples of yeast juice, dried yeast and zymin, using both fructose and glucose but otherwise keeping the conditions as similar as possible. The products were worked up according to a definite scheme, somewhat modified from the original method [Robison, 1922] as further experience had suggested. The separate experiments were carried out using 50 g. of dried yeast or zymin, or 300 cc. of yeast juice, to which were added 100 g. glucose or fructose, and sodium phosphate equivalent to about 7 g. P. The phosphate solution was added in six equal portions over a period of 5 to 6 hours, as indicated by the rate of evolution of carbon dioxide. When, after the last addition, the rate had again fallen, trichloroacetic acid was added to the fermentation mixture in amount equal to 4 % of the total volume. The precipitated proteins were filtered off and the filtrate treated with barium acetate equal in weight to the crystalline sodium phosphate originally added, followed by baryta in hot aqueous solution until pink with phenolphthalein. The precipitate consisting of crude barium hexosediphosphate was removed and a further precipitate, obtained by adding one-tenth volume of alcohol to the filtrate, was likewise filtered off. The solution containing the soluble barium salts was treated with basic lead acetate, and the precipitated lead salts decomposed with hydrogen sulphide. After removal of the excess of hydrogen sulphide by aeration, the acid solution was neutralised with baryta and poured into 3 volumes of alcohol. By extracting the fraction with 10 parts of 10 % aqueous alcohol the readily soluble barium salts were freed from a further quantity of sparingly soluble compounds.

All fractions obtained at each step in the separation were quantitatively examined, the total and inorganic phosphorus, the reducing power by the Hagedorn and Jensen and by the sodium hypoiodite methods, and the specific rotation being measured.

On comparison of the results obtained for the whole series of experiments a very striking difference was seen between the products of fermentation with yeast juice on the one hand and with dried yeast and zymin on the other. In the experiments with yeast juice the sparingly soluble barium hexosediphosphate represented less than half the total esterified phosphorus, while

the properties of the large, readily soluble fraction were similar to those of the barium hexosemonophosphate previously described. As in the earlier experiments fractions of intermediate solubility were also obtained and these are being investigated. In the experiments with dried yeast and zymin a much higher proportion of diphosphate was obtained, this fraction representing between 80 and 90 % of the total esterified phosphorus. Further, the small fraction of readily soluble salt differed markedly in its properties from barium hexosemonophosphate, as is shown in Table I, in which the results of a number of experiments with dried yeast and zymin are summarised. One yeast juice experiment is included for purposes of comparison.

Table I. *Readily soluble fraction of barium salts.*

Exp.	Type of yeast preparation*	Sugar	Total P in fraction as percentage of total esterified P†	P %	Reducing power as glucose: H. & J %	Reducing power as glucose: Iodine %	$[\alpha]_{5461}$	Reduction P	$[\alpha]_{5461}$ P
6	Dried yeast	Fructose	5·9	5·5	8·3	12·5	+56·7°	1·5	10·4
7	,,	,,	11·7	6·3	13·6	15·6	+41·4°	2·2	6·6
8	,,	Glucose	10·7	5·9	11·0	14·1	+56·4°	1·9	9·6
9	,,	Fructose	6·4	5·3	5·5	10·8	+63·8°	1·0	12·0
15	Zymin	,,	7·5	6·7	18·1	20·0	+28·0°	2·7	4·2
16	,,	Glucose	4·3	6·2	16·2	19·0	+30·0°	2·6	4·8
13	Yeast juice	Fructose	47·3	7·4	27·4	23·4	+11·4°	3·7	1·6

* Exps. 6, 7 and 8 were carried out with English brewery top yeast. In Exp. 9 a bottom yeast obtained from the Standard Yeast Co. was used.

† A small quantity of organic phosphorus remained in the final filtrates from the basic lead and barium salts. Some of this could be recovered in a very impure condition by treatment of these filtrates with basic lead acetate and ammonia. These amounts, if credited to the most soluble fraction, would increase the figures shown in the above table by several per cent.

Isolation of the new phosphoric ester.

The values shown in Table I indicated that the soluble fractions from the dried yeast and zymin fermentations contained a compound possessing a much higher dextro-rotation and lower reducing power than those of barium hexosemonophosphate. It was found that on fractional precipitation of the aqueous solution by alcohol the specific rotation of the more soluble portion was still further raised and the reducing power lowered. Accordingly a quantity of the crude soluble barium salts (about 50 g.) obtained from 5 experiments (Nos. 1–5), in all of which dried English top yeast and fructose had been used, was dissolved in 10 parts of water and treated with alcohol, raising the concentration of the latter successively to 10, 17, 23, 30 and 70 %. The precipitates obtained between 10 and 30 % alcohol were oily in nature and as the solubility of these salts increased with the temperature it was found convenient to heat the solution to about 50° before adding the alcohol, whereby the separation of an oily phase took place slowly during cooling to room temperature. After standing for some hours the clear supernatant solution could be readily poured off, again warmed and treated with a further quantity of alcohol. No advantage was gained by cooling the solution to 0° or by separating the fraction at 37°.

The small flocculent precipitate obtained on raising the concentration of alcohol from 30 to 70 % possessed a high dextro-rotation and very low reducing power. After many repetitions of this process a quantity of barium salt was obtained which possessed a reducing power (H. and J.) of only 1 % in terms of glucose whilst the specific rotation had risen to over + 100°. Since continued fractionation by alcohol did not further lower the reducing power the barium salt was converted into the brucine salt which unexpectedly proved to be very sparingly soluble in cold water and readily crystallisable from hot aqueous solution. After several recrystallisations it was reconverted into the barium salt which now showed no reducing power either by the Hagedorn and Jensen or the sodium hypoiodite method. The analytical figures for this salt agreed with those required for a disaccharide monophosphate, while the properties of the ester, its resistance to acid hydrolysis, negative Selivanov's reaction and the very high specific rotation ($[\alpha]_{5461} + 132°$) suggested that it was a derivative of trehalose. This supposition was confirmed by investigation of the products of hydrolysis of the ester by acids and by the bone phosphatase, the former yielding glucose and glucosemonophosphoric ester, while the latter yielded trehalose itself which was isolated in pure crystalline form.

Brucine salt of trehalosemonophosphoric ester.

The barium salt obtained from the crude soluble fraction by repeated precipitation with alcohol (30 to 70 % fractions) was dissolved in five parts of water and treated with the theoretical quantity of sulphuric acid. After removal of the barium sulphate by centrifuging, brucine (2 mols.) was added and the solution treated with an equal volume of acetone. The brucine salt crystallised out in the course of a few hours. It was recrystallised several times from hot water, from which it separated in large clusters of needles. It is very slightly soluble in hot ethyl alcohol, somewhat more soluble in methyl alcohol, insoluble in chloroform and acetone.

Analysis. For analysis the salt was dried over sulphuric acid at room temperature. The water of crystallisation was estimated by heating over P_2O_5 at 100° and 15 mm. until the weight was constant (2 hours). P was estimated by the Briggs method; C and H by Pregl's micro-method.

	H_2O	C	H	P
Found	11·8 11·7	50·9	6·8	2·31
Calculated for $C_{12}H_{21}O_{10}PO_4H_2(C_{22}H_{26}O_4N_2)_2 9H_2O$	11·66	50·73	6·82	2·26

$[\alpha]^{20°}_{5461}$ in water ($c = 0{\cdot}77$ %) + 31·3°

$[\alpha]^{20°}_{5461}$ for the anhydrous salt + 35·4°

Barium trehalosemonophosphate.

The pure brucine salt was dissolved in warm water and treated with a cold aqueous solution of baryta until pink with phenolphthalein. The barium salt was precipitated by the addition of 3 volumes of alcohol, filtered, washed with absolute alcohol and dried quickly in a vacuum desiccator. To remove traces

of brucine the salt was dissolved in 5 parts of water, reprecipitated with alcohol, filtered and dried as before, and the process repeated. The salt so prepared is a white amorphous powder, stable in the air, but like hexosemonophosphate turning syrupy with small quantities of water, in which it is very soluble. It is insoluble in absolute alcohol but in aqueous alcohol it is decidedly more soluble than barium hexosemonophosphate, the solubility increasing with the temperature. At 22°, 100 cc. of 30 % (by vol.) alcohol dissolves about 1 g. while the 75 % alcohol filtrates still contain appreciable traces of the salt. Unlike the hexosephosphates it can be heated to above 100° in the air or *in vacuo* without becoming discoloured, this stability being probably connected with absence of a reducing group. At 130° it slowly turns yellow. It does not reduce Hagedorn and Jensen's solution and gives no coloration with Selivanov's reagent for fructose.

Analysis. For analysis the salt was dried at 100° and 15 mm. over P_2O_5, or in Pregl's micro-desiccator at 120°, until the weight remained constant. *a*, *b* and *c* were different specimens, but were all derived from the same 50 g. of crude soluble salts. The results do not at present justify a definite conclusion as to the presence of a molecule of water in the salt.

	C	H	P	Ba
Found (*a*)	25·5	4·0	5·43	23·6
	25·5	4·0	—	—
(*b*)	25·9	4·3	5·35	24·4
(*c*)	25·7	4·2	5·51	23·4
	25·6	4·1	5·49	23·4
	25·5	4·1	—	23·5
	25·6	4·1	—	—
Calculated for $C_{12}H_{21}O_{14}PBa$	25·82	3·80	5·57	24·63
Calculated for $C_{12}H_{21}O_{14}PBa + H_2O$	25·01	4·03	5·39	23·84
$[\alpha]^{20°}_{5461}$ of the salt in water ($c = 3·2$ %)	+132°			
$[\alpha]^{20°}_{5461}$ of the free acid in water ($c = 2·4$ %)	+185°			
$[M]^{20°}_{5461}$ of the free acid (M = 422)	+781°			

Hydrolysis of trehalosemonophosphoric ester by bone phosphatase.

A preliminary experiment showed that bone phosphatase removed the phosphoric acid group of the new ester but was without action on the liberated disaccharide. Accordingly 380 mg. of the dry barium salt dissolved in 10 cc. water was treated with purified bone phosphatase (to be described shortly in a paper by Martland and Robison) and kept at 40°, the p_H being maintained at 8·6–8·8 by the addition of baryta at frequent intervals. Hydrolysis was complete in about 10 hours and alcohol (3 vols.) was then added to precipitate the enzyme and any barium salts remaining in solution. The alcoholic filtrate was evaporated in a vacuum desiccator and the syrupy residue taken up in methyl alcohol. The solution was filtered and evaporated to dryness, the residue dissolved in a few cc. of water and the solution again filtered and evaporated.

Weight of residue, 169 mg. representing 73 % of the theoretical yield of trehalose. It contained no phosphorus, was non-reducing, and had $[\alpha]_{5461}^{20°} + 196°$.

The product was further purified by recrystallisation from hot alcohol from which it separated in lustrous rhombic prisms, M.P. 96°–98° (uncorr.). This figure is in agreement with the M.P. of hydrated trehalose given by Schukoff [1900]. Koch and Koch [1925] give a somewhat higher M.P., 102·5°. The solubilities of the crystals in water and alcohol also agreed with those of trehalose.

Analysis. For analysis the air-dried crystalline substance was used.

	C	H
Found	38·1	6·8
	38·0	6·8
Calculated for $C_{12}H_{22}O_{11}, 2H_2O$	38·1	6·9
$[\alpha]_{5461}^{19°}$ in water ($c = 0·2$ %)	$+222°$	

The values of $[\alpha]_D$ of trehalose recorded in the literature vary between + 173° (for the hydrated sugar) [Mitscherlich, 1858] and + 184° [Koch and Koch, 1925]. No figures for $[\alpha]_{5461}$ have been found, but Koch's figure for $[\alpha]_D$ multiplied by the factor 1·18 gives $[\alpha]_{5461} + 217°$. Calculated from this figure the molecular rotation of trehalose is $[M]_{5461} + 820°$, while that found for the free trehalose-monophosphoric ester was + 781°. The effect of the introduction of the phosphoric acid group on the rotatory power of the molecule is thus relatively small.

Hydrolysis by acids. The disaccharide was very resistant to acid hydrolysis. 16 mg. of the crystalline sugar were dissolved in 10 cc. 2 *N* H_2SO_4 and heated at 100° until the rotation became approximately constant (5 hours). The initial and final values observed for rotation and reducing power are shown below:

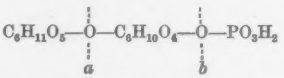
		Reducing power (mg. glucose in 10 cc.)	
Time (hours)	α_{5461} ($l = 1$)	H. and J.	Iodine
0	+0·35°	0	0
5	+0·13°	13·6	13·2
Percentage of total change calculated for conversion to two molecules of glucose	86	88	86

An osazone was prepared from the hydrolysis product, which, after recrystallisation from dilute pyridine, melted at 202°–204° and did not lower the melting-point when mixed with a specimen of glucosazone. The properties of the disaccharide obtained from the ester by enzymic hydrolysis thus agreed in all respects with those of trehalose.

Hydrolysis of trehalosemonophosphoric ester by acid.

Trehalosemonophosphoric ester is slowly hydrolysed by dilute mineral acids at 100°, the ultimate products being glucose and phosphoric acid. Since

the rate of hydrolysis of the phosphoryl linkage "*b*" is very much slower than that of the glucosidic linkage "*a*"



glucose and glucosemonophosphoric ester at first accumulate and the latter can be isolated by stopping the hydrolysis at a suitable stage. Owing to the small quantity of material available, the glucosemonophosphate has not yet been obtained in absolutely pure condition but it will be interesting to discover whether, as appears likely, it is identical with one constituent of hexosemonophosphate (Robison).

Table II.

Hydrolysis of trehalosemonophosphoric ester (*M*/20 sol.) by 0·2 *N* H_2SO_4 at 100°.

	Degree of hydrolysis as measured by			
		Reducing power		
Time (hours)	Inorganic P set free %	H. and J. %	Iodine %	Fall in rotation %
5	3	18	22	19
10	9	34	40	39
21	18	64	71	73
40	36	82	93	93
58	51	86	97	100
67	53	84	100	101

Table II shows the progress of one hydrolysis experiment with 0·2 *N* acid. Even after 67 hours only 53 % of the phosphoric acid had been set free. The reducing power towards an alkaline solution of iodine is a measure of the free aldose group and the figures in this column show that hydrolysis of the glucosidic linkage was complete. The lower values obtained by the Hagedorn and Jensen method are due to the presence of glucosemonophosphate which has only two-thirds the reducing power of the equivalent weight of glucose. The fall in rotation, also calculated as a percentage of the total change for complete hydrolysis to glucose, is seen to follow very closely the figures for the reducing power towards iodine, showing that the molecular rotation of the glucosemonophosphoric ester is very similar to that of glucose.

The results of another acid hydrolysis with *N* H_2SO_4 are shown in Fig. 1, the parallelism between the increase in reducing power towards iodine (curve *C*) and fall in rotation (curve *D*) being again very marked. The divergence of the two curves towards the end of the experiment is due to the destruction of a little glucose by the acid which increases the apparent hydrolysis as measured by the fall in rotation. This also explains the fall in the reducing power by the Hagedorn and Jensen method. In this experiment the hydrolysis was stopped after 21 hours and the acid solution neutralised with baryta. After removal of the barium sulphate the solution was concentrated and poured into twice its volume of alcohol to precipitate the barium glucosemonophosphate. The

alcoholic filtrate on evaporation yielded a reducing sugar having all the properties of glucose.

Weight of product	0·524 g.
Reducing power as glucose (Hagedorn and Jensen)	0·393 g.
Reducing power as glucose (Iodine)	0·384 g.
$[\alpha]_{5461}$ calculated on the glucose content as estimated by Hagedorn and Jensen's method	+ 61°
For glucose $[\alpha]_{5461}$	+ 62°

Osazone: M.P. 204°, not lowered by admixture with pure glucosazone.

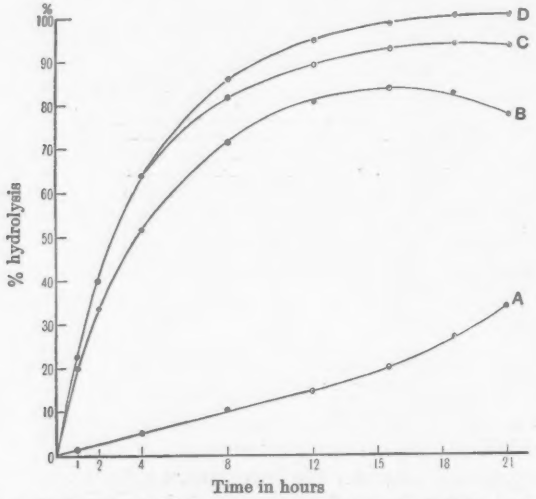


Fig. 1. Hydrolysis of trehalosemonophosphoric ester by N H_2SO_4 at 100°.

Curve *A*. Inorganic P liberated.
Curve *B*. Reducing power by Hagedorn and Jensen method.
Curve *C*. Reducing power by the sodium hypoiodite method.
Curve *D*. Change in rotation.

The points on the curves represent in each case percentages of the total values calculated for complete hydrolysis of the ester to glucose (2 mols.) and phosphoric acid.

The alcohol precipitates obtained from this and other hydrolysis experiments were separately purified by solution in 10 % alcohol, and precipitation by addition of twice the volume of alcohol. Even after several repetitions of this process the salt still contained a small proportion of sparingly soluble substance. It was difficult to account for the persistence of this impurity which may have been due to oxidation or other change of the monophosphate. The figures for this compound must therefore be given subject to future correction.

Barium glucosemonophosphate.

Preparation	Total P %	Reducing power as glucose: H. and J. %	Reducing power as glucose: Iodine %	$[\alpha]_{5461}$
a	7·63	27·9	35·6	+20°
b	7·40	32·0	42·0	+23·5°
c	7·33	31·0	42·4	+21°
Calculated for barium glucosemonophosphate	7·85	(?32)	45·5	—

Fermentation of trehalosemonophosphate.

Experiments were made to determine whether trehalosemonophosphoric ester is fermented by yeast juice, dried yeast and zymin. A solution of the sodium salt (sol. *a*) was used, of such strength that 1 cc. was equivalent to 30 mg. glucose, and control tests were also made to determine (*b*) the rate of fermentation of sucrose (sol. *b*, 1 cc. = 40 mg. sucrose), and (*c*) the autofermentation rate of the yeast preparation.

1 cc. sol. *a* should give about 8 cc. carbon dioxide on complete fermentation.
1 cc. sol. *b* should give about 11·2 cc. carbon dioxide on complete fermentation.

The results show that the ester was readily fermented by dried yeast, the rate of decomposition being even more rapid than in the case of sucrose. It was also fermented, although more slowly, by zymin and by yeast juice.

Exp. 1. (*a*) 0·2 g. dried yeast + 1 cc. water + 1 cc. sol. *a* + 1 drop toluene.
(*b*) 0·2 g. dried yeast + 1 cc. water + 1 cc. sol. *b* + 1 drop toluene.
(*c*) 0·2 g. dried yeast + 2 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved: (*a*)	(*b*)	(*c*)
0	0·0	0·0	0·0
½	1·70	0·71	0·30
1	3·45	1·70	0·71
1½	4·40	2·30	0·80
3	5·69	3·59	0·90
5	5·91	5·35	0·95
22	6·47	6·66	0·95
Total corrected for autofermentation (*c*)	5·52	5·71	—

Exp. 2. (*a*) 0·2 g. zymin + 1 cc. water + 1 cc. sol. *a* + 1 drop toluene.
(*b*) 0·2 g. zymin + 1 cc. water + 1 cc. sol. *b* + 1 drop toluene.
(*c*) 0·2 g. zymin + 2 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved: (*a*)	(*b*)	(*c*)
0	0·0	0·0	0·0
1	3·75	4·36	3·15
2¾	6·90	11·36	6·30
18	11·97	17·86	7·42
Total corrected for autofermentation (*c*)	4·55	10·44	—

Exp. 3. (a_1) and (a_2) 1 cc. yeast juice + 1 cc. sol. a + 1 drop toluene.
(c) 1 cc. yeast juice + 1 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved (a_1)	(a_2)	(c)
0	0·0	0·0	0·0
½	0·63	0·68	0·37
1	1·37	1·39	0·98
1½	2·07	2·17	1·50
15	6·65	7·01	2·37
Total corrected for autofermentation (c)	4·28	4·64	—

Examination of dried yeast for trehalose and trehalosemonophosphate.

Trehalose has been isolated from dried yeast by Koch and Koch [1925]. These workers obtained the sugar by extracting 40 lb. of air-dried yeast with 90–95 % alcohol but give no information as to the yield. From the data of our experiments it may be reckoned that the amount of trehalosemonophosphoric ester present in the fermentation products from 50 g. dried yeast, corresponded with at least 100–300 mg. P, *i.e.* about 1–3 g. of trehalose. It was not impossible that such amounts of the ester were originally present in the yeast or were produced during the fermentation by simple esterification of preformed trehalose. Experiments were carried out to test these possibilities.

(*a*) *Presence of trehalose in dried yeast.* 10 g. dried yeast was extracted for 8 hours with boiling ether to remove fat. It was then extracted with three successive quantities of boiling 96 % alcohol for 24 hours in all. The residues obtained on evaporating the three alcoholic extracts weighed 445 mg., 59 mg. and 38 mg., making a total of 542 mg. The rotation and reducing power of this residue before and after acid hydrolysis (2 *N* HCl, 100°, 6 hours) are shown below:

542 mg. in 30 cc. 2 *N* HCl	Before hydrolysis	After hydrolysis
a_{5461} ($l = 1$)	−0·02°	−0·04°
Total reducing power as glucose	18 mg.	37 mg.

Even assuming that the increase in reducing power on hydrolysis was entirely due to trehalose, the amount of this sugar present in the yeast could not be more than about 0·2 %, equivalent to 100 mg. trehalose in 50 g. dried yeast. Other samples of dried yeast used in the fermentation experiments gave similar results.

(*b*) *Presence of trehalosemonophosphoric ester in dried yeast.* 50 g. of the dried yeast used in the fermentation experiments Nos. 1–5 were extracted with 250 cc. of 4 % trichloroacetic acid for 3 hours at room temperature. The extract, which contained 198 mg. inorganic P and 552 mg. organic P, was filtered and neutralised with baryta. The precipitated barium salts contained the whole of the inorganic P and over 92 % of the organic P, a large proportion of the latter being present as nucleic acid. Some hexosediphosphate was also present. The readily soluble fraction, in which barium trehalosemonophosphate would be found, contained only 15 mg. phosphorus and gave a strongly

laevorotatory solution. Two similar extractions of other dried yeast preparations gave very similar figures showing that trehalosemonophosphoric ester, if present in the original dried yeast, can only occur in very small amounts.

Discussion.

From the experimental results given above it must be concluded that the trehalosemonophosphoric ester isolated from the products of the fermentation of fructose by dried yeast was not originally present in the yeast, nor could it have been derived to any great extent from preformed trehalose. The occurrence of a trehalase in various types of yeast has been observed [Fischer, 1895; Kalanthar, 1898; Bau, 1899; Lindner, 1911] and by the synthetic action of this enzyme, trehalose could be formed from glucose derived from the yeast glycogen. It is unlikely, however, that the dried yeast contained sufficient glycogen to account for more than a small fraction of the trehalosephosphoric ester, nor is this necessary since there is sufficient other evidence of the formation of glucose derivatives during the fermentation of fructose.

Trehalosemonophosphoric ester has not so far been found in the products of fermentation of yeast juice. It appears to be formed equally in the fermentation of fructose or glucose by dried yeast and also by zymin. (The investigation of the zymin products is not yet complete.) Using the same dried yeast preparation but varying the conditions of fermentation we have been able to modify the amount and nature of the soluble fraction of barium salts and are continuing the investigation on these lines.

It would be premature to venture an opinion as to whether the formation of this disaccharide phosphoric ester has any great significance in the carbohydrate metabolism of the yeast, but it is not impossible that the introduction of the phosphoric acid group may facilitate the building up of the complex reserve carbohydrates from the simple sugars.

Neuberg and Leibowitz [1928] have recently reported the preparation of another disaccharide monophosphoric ester by the action of *B. delbrücki* on hexosediphosphate. This ester, which was obtained in a yield of 30 %, possessed reducing properties and $[\alpha]_D + 38°$ for the barium salt. It is therefore not identical with the ester described in this paper. A synthetic trehalosemonophosphate has been prepared by Helferich, Löwa, Nippe and Riedel [1923] by the action of phosphoryl chloride on trehalose in pyridine solution. No properties are given other than the specific rotation of the acid barium salt, $[\alpha]_D + 135{\cdot}5°$, and it is therefore impossible to decide whether their compound was identical with the ester which has now been isolated from the products of fermentation.

SUMMARY.

1. A monophosphoric ester of the disaccharide trehalose has been isolated from the products of fermentation of fructose by dried yeast, and has been purified by means of its barium salt, which is very soluble in water, and its crystalline brucine salt.

2. The ester is non-reducing and is strongly dextrorotatory: $[\alpha]_{5461}$ of the free acid + 185°, of the barium salt + 132°, and of the brucine salt + 31°.

3. By the action of the bone phosphatase on the ester pure crystalline trehalose has been obtained.

4. Hydrolysis with boiling mineral acid slowly converted the ester into glucose and glucosemonophosphoric ester, the latter being still more slowly hydrolysed to glucose and phosphoric acid. Barium glucosemonophosphate was isolated but in a slightly impure condition.

5. Trehalosemonophosphoric ester is readily fermented by dried yeast and more slowly by yeast juice and zymin.

6. Examination of the dried yeast used for the fermentation showed that neither trehalosemonophosphoric ester nor free trehalose was present in quantities sufficient to account for the amount of the ester found in the fermentation products.

7. No evidence has been obtained of the presence of this ester in the products of fermentation by yeast juice.

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CLX. THE CARBOHYDRATE METABOLISM OF CERTAIN PATHOLOGICAL OVERGROWTHS.

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(*Received August 25th, 1928.*)

An important biochemical aspect of the cancer problem has been stressed by Warburg and his collaborators (1924–1927) in their study of the carbohydrate metabolism of surviving tissues, normal and pathological.

An enquiry into the problem whether the basic chemical reactions which yield energy for the maintenance and growth of cells are the same in kind and velocity for normal and abnormal tissues has resulted in establishing the fact that these tissues exhibit fundamental differences in their manner of utilising carbohydrates.

Warburg has shown that malignant tissues, like certain varieties of yeasts, possess the dual capacity to metabolise carbohydrates by oxidative and splitting processes.

Normal growing tissues, while using the oxidative process (respiration) almost exclusively under *aerobic* conditions, have the power of splitting carbohydrate to lactic acid under *anaerobic* conditions (glycolysis).

The characteristic property of the cancer cell is its power and habit, even under *aerobic* conditions, to metabolise carbohydrate by both these processes.

That the energy freed by the glycolytic process (amounting, in the case of tumour tissue to about 35–40 % of that freed by respiration) is utilised in the cell economy, cannot be definitely established, but experiments on survival of tumour cells *in vitro* by Okamoto [1925] suggest that this is the case, while Warburg's studies on the inhibiting effect of narcotics on the glycolytic functioning of tumour cells would seem to show that glycolysis is a reaction associated with cell structures, the liberated energy being at the disposal of the cell.

In this connection the following questions arise. (1) Is this peculiarity of metabolism an exclusive feature of malignant tissue? (2) Are there quantitative relationships between the magnitudes of the respiration and the *aerobic* and *anaerobic* glycolyses, which afford a means of differentiating normal from pathological tissues?

Warburg has, at various times, suggested the following generalisations. (*a*) The ratio $\frac{\text{aerobic glycolysis}}{\text{respiration}}$: this was found to be approximately three for

tumour tissue, and small or zero for normal tissues; (*b*) the value U, or excess fermentation, based upon the assumption that the Pasteur reaction is functioning at its maximum efficiency. U would then represent the minimum theoretical value for the aerobic glycolysis. For tumour tissue, this expression was found to be positive, for normal tissues, negative.

Many facts have been brought forward, which suggest that such attempts to differentiate malignant tissues, with respect to their carbohydrate metabolism, from normal resting or growing tissues are not fully justified.

Murphy and Hawkins [1925] found great variability in their results. Rat placenta, according to these authors, behaved as a malignant tissue, and many spontaneous mouse tumours fell into the category for normal resting tissues. Pentimalli [1927], regarding tumours as the result of a repeatedly disturbed regeneration process, found that regenerating muscle in the abdominal wall of young chicks behaved, with respect to its glycolytic metabolism of carbohydrates, qualitatively in the manner of tumour tissue, though quantitatively the results were considerably smaller. Fleischmann and Kubowitz [1927] found that the leucocytes of geese show a carbohydrate metabolism similar to that of tumours. Warburg [1927] records the fact that red blood corpuscles also possess a positive U value, and suggests that this anomaly may be due (as also with white blood cells) to the fact that they are free-living cells which are nearing the end of their life-cycle in the circulation. Warburg, Krebs [1927] and Tamiya [1927] have found that the retina is an exceptional tissue, since it possesses a glycolytic capacity of greater magnitude than that of any other animal tissue. In this case Warburg has introduced a new conception, showing acceleration or retardation of anaerobic glycolysis with the progressive development of the tissue, to differentiate tumour from retinal metabolism.

For normal tissues the value $\frac{dQ^{N_2}_{M}}{dt}$ (see p. 1291) is negative, for tumour tissues zero, and for retina tissues positive.

It appeared of interest, in the light of extrinsic hypotheses on the cause of carcinogenesis, to study the carbohydrate metabolism of overgrowths associated with recognised viruses, and accepted generally as being devoid of the essentials of malignancy.

The lesions of fowl pox in pigeons, vaccinia lesions in young chickens, vaccinia lesions in rabbits, and human warts have been examined up to the present time. A study of the metabolism of brain tissue of cavies dying of rabies has also been made, where an intracellular virus is active, but unaccompanied by any tissue proliferation. The Rous sarcoma has also been examined.

Where proliferation has taken place and a gross lesion is manifested, the carbohydrate metabolism was found to be greatly enhanced, and in a manner analogous to that of tumours. Where no hyperplasia was present, no deviation was found from the normal values for the tissue concerned.

Technique and expression of results.

The manometric technique elaborated by Warburg has been followed throughout. It is fully described in Warburg's collected papers [1926]. Any variations from the standard methods of determination of the metabolism quotients are referred to under separate headings.

The mode of expressing results is that used by Warburg. Simultaneous measurements are made of respiration, aerobic and anaerobic glycolysis, and their magnitudes expressed as the number of mm.^{3} of gas consumed or evolved per mg. of dried tissue per hour.

$$Q_{O_2}\ \text{(respiration)} = \frac{\text{mm.}^3\ \text{oxygen consumed}}{\text{hours} \times \text{mg. dry tissue}}.$$

$$Q_M^{O_2}\ \text{(aerobic glycolysis)} = \frac{\text{mm.}^3\ CO_2\ \text{evolved by lactic acid formation in } O_2}{\text{hours} \times \text{mg. dry tissue}}.$$

$$Q_M^{N_2}\ \text{(anaerobic glycolysis)} = \frac{\text{mm.}^3\ CO_2\ \text{evolved by lactic acid formation in } N_2}{\text{hours} \times \text{mg. dry tissue}}.$$

Fowl-pox.

In measuring the metabolism quotients of these lesions, certain inherent difficulties arise, which are not met with in the more straightforward cases of transplanted animal tumours or normal animal tissues. In order to get the maximum effects *in vitro* of dissolved gaseous and solid metabolites, perfect diffusion of these to the cells most remote from the surface must be ensured. Under the experimental conditions used, it can be calculated theoretically that the maximum thickness of the tissue slices must be no greater than 0·5 mm. Owing to the fatty nature of these lesions, the practical difficulties of obtaining such thin sections are great, with the result that the quotients obtained are probably less than the true values which would be found under perfect conditions of diffusion. These lesions moreover are not homogeneous in character, and allowances must obviously be made on this account.

The sections were prepared by cutting perpendicularly to the skin surface, and, on the average, each section had an area of approximately 0·5 cm.^{2}, and a dry weight of 15–30 mg. A histological examination showed a greatly thickened and proliferated epidermal layer, a high percentage of fat, connective tissue, and occasionally a few muscle cells.

The muscle cells, as shown by Meyerhof [1921], Pentimalli [1927], and controls quoted later for Rous tumours, show a very low carbohydrate metabolism under the experimental conditions. Figures quoted by Warburg [1926] indicate that connective tissue shows a negligible metabolism, and it seems probable that the fat makes no contribution to the results obtained.

On the basis of both histological and chemical estimations of fat, a minimum of 50 % of the total dry weight was found. It seems justifiable, therefore, to multiply the actual figures obtained by at least 2, in calculating the metabolism of the proliferating epithelium. The following table of results shows the experimentally obtained quotients for a series of lesions at their maximum phase of hyperplasia.

Table I. *Carbohydrate metabolism quotients of fowl-pox lesions.*

No. of days after transmission	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Meyerhof quotient	U
10	−3·3	+4·7	+10·0	1·6	+3·4
9	−3·8	+5·1	+11·8	1·7	+4·2
10	−4·4	+6·5	+12·3	1·3	+3·5
9	−4·8	+5·2	+ 8·4	0·7	−1·2
9	−1·1	+3·3	+10·0	—	+7·8
7	−6·0	+5·2	+ 8·5	0·6	−3·5
5	−3·2	+5·5	+11·3	1·8	+4·9
8	−4·3	+5·9	+12·4	1·5	+3·8
13	−3·1	+2·8	+ 8·3	1·8	+2·1
11	−7·8	+8·5	+18·0	1·2	+2·4
15	−2·1	+2·9	+ 7·3	2·1	+3·1

The measurements were made in Ringer solution, containing glucose and sodium bicarbonate, at 37·8°, the gas phase being 5 % CO_2 in oxygen for aerobic, and 5 % CO_2 in nitrogen for anaerobic measurements.

The composition of the saline medium used was:

Salt	Moles per litre
NaCl	0·121
KCl	0·0025
$CaCl_2$	0·0018
$NaHCO_3$	0·0025
Glucose	0·2

In dealing with sections composed of different types of tissue, it is clear that the aerobic values obtained (dependent upon a calculation from the observed gas-exchange in two vessels containing different amounts of nutrient media) would only be valid if the sections used were entirely similar in composition. This condition is difficult to obtain, and two methods have been used to minimise errors arising from this:

(*a*) measurements were made with adjacent sections;

(*b*) measurements were made with the same section, making consecutive observations in different volumes of Ringer solution.

The results obtained have been similar by both methods. These results, making a minimum allowance of 50 % for the fat included in this dry weight, may be summarised as follows:

Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	U
−6·6 to −15·6	+9·4 to +17·0	+20 to +36	1·2 to −2·1	+4·8 to +15·6

A comparison of these values with those obtained by Warburg for animal, human and avian tumours, shows them to be of the same order of magnitude, and to possess the same relationships to one another. In particular, the excess fermentation, U, is positive in nearly every case, making these lesions fall into the category of malignant tumours, if the most recent classification of Warburg be adopted.

Certain results obtained with embryonic tissue and liver show different values for the aerobic glycolysis when measured in serum from those obtained

by measurements in Ringer. Warburg attributes this to the extreme sensitiveness of the Pasteur reaction, the respiratory activity failing to function effectively owing to rapid damaging of tissue placed in Ringer solution. Hence the metabolism of fowl-pox lesions was measured in serum, both foreign (inactivated horse-serum) and autologous serum (pigeon).

Table II gives the results of a series of experiments, and indicates that the change of medium has little effect on the metabolism quotients.

Table II.

Serum	No. of days after transmission	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	U
Pigeon	4	−1·9	+ 4·8	+ 7·0	1·2	+ 3·2
	10	−4·6	+12·9	+20·5	1·65	+11·3
	11	−2·1	+ 9·1	+16·3	3·0	+12·1
	10	−4·2	+13·0	+20·5	1·8	+12·1
	13 (regressing)	−1·0	+ 2·7	+ 7·5	—	+ 5·5
Inactivated horse	7	−4·0	+ 3·3	+ 8·2	1·2	+ 0·2
	6	−3·4	+ 2·8	+ 7·3	1·3	+ 0·5
	9	−3·1	+ 5·8	+ 9·6	1·2	+ 3·4
	10	−4·2	+ 5·9	+11·3	1·3	+ 2·9

In order to see if there was a correlation between the metabolism and the progressive epithelial hyperplasia, a series of measurements was made from day to day of the developing lesions. The results are represented graphically in Fig. 1.

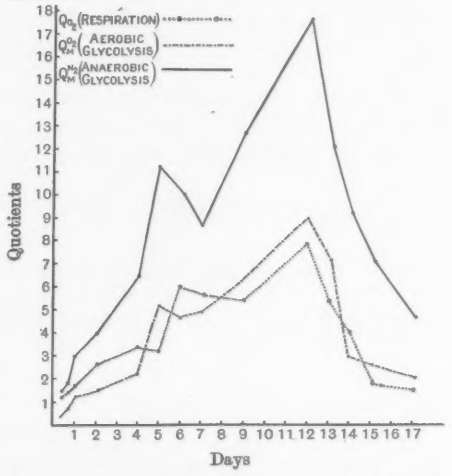

Fig. 1. Curves showing the changes in the carbohydrate metabolism of fowl-pox lesions, during development and regression.

Normal pigeon skin gave low values for all the quotients,

$$e.g.\ Q_{O_2} = -0{\cdot}9;\ Q_M^{O_2} = +0{\cdot}5;\ Q_M^{N_2} = +1{\cdot}7.$$

As the lesion attained its maximum growth (12 or 13 days), the respiration, aerobic and anaerobic glycolysis increased, and with its slow regression all these quotients decreased to the level typical of normal skin.

To account for the aerobic glycolysis found in red and white blood cells, Warburg suggests that they are in a degenerating condition. The progressive character of the metabolism with proliferation of the epithelium in fowl-pox lesions, where histological examination shows numerous mitotic figures, suggests that the concept of degeneration does not account for such increases as are observed. Moreover, when regression commences, and it would be expected that degeneration would follow, the glycolytic metabolism tends to disappear.

Vaccinia.

(*a*) *Rabbit.* The vaccinia lesion consists of dense infiltration of the dermis with polymorph leucocytes. The adventitial or reticulo-endothelial cells are numerous and in active mitosis. There is no marked change in the Malpighian layer, except early chromatolysis and swelling. Mitotic figures are occasionally seen.

Table III shows the quotients obtained in two experiments with these lesions, and with normal rabbit skin.

Table III.

Tissue	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Vaccinia lesion (rabbit)	−3·3	+2·3	+2·8
	−3·0	+2·0	+2·7
Normal rabbit skin	−1·0	+1·3	+1·9

Sections perpendicular to the skin surface were used and the measurements made in Ringer solution containing glucose and bicarbonate. The figures refer to the whole lesion, with no allowances for material of very small, or no, measurable metabolism.

The increase in metabolic activity is small, and might be attributed to the presence of leucocytes.

(*b*) *Young chickens.* The lesions produced in one to seven days old chickens by the vaccinia virus are identical histologically with those produced by the fowl-pox virus, as shown by Findlay [1928] and Ludford [1928].

Table IV.

Tissue	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	U
Vaccinia lesion	−5·0	+4·2	+11·1	1·4	+1·1
	−3·2	+3·6	+ 7·8	1·3	+1·4
	−4·8	+4·0	+10·6	1·4	+1·0
Normal chicken skin	−1·0	+0·4	+ 1·5	—	—

Corresponding to the epithelial hyperplasia, a great increase in the carbohydrate metabolism is noticed (Table IV).

The measurements were made in Ringer solution, and the figures represent the metabolism of the whole lesion. The same difficulties of obtaining sections of the necessary thickness for perfect diffusion of metabolites and the presence of non-reacting tissues make the values quoted minimal.

Human warts.

The metabolism quotients found in the three cases examined are much smaller than those found for fowl-pox lesions (Table V).

The gas exchanges observed during the experiments were comparatively large, and suggest an active metabolism for the relatively small percentage of physiologically active epithelial cells present. Considerably more than half of the section was composed of fully keratinised epithelium and connective tissue, giving the dry weight a value many times greater than that of the reacting portion.

Table V.

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	U
A	−1·7	+2·2	+4·1	1·1	+0·7
B	−1·2	+1·7	+3·9	1·8	+1·5
C	−1·2	+1·6	+3·8	1·8	+1·4

Rabies.

The strain of virus used was a "virus fixe" which is highly pathogenic for guinea-pigs, and on intracerebral inoculation produces a fatal result in a week. The animals were killed when moribund and the brain removed for examination.

Sections were cut tangentially from the cerebral cortex and hippocampal region.

The average quotients obtained for the outer layer of normal guinea-pig cerebral cortex were:

$$Q_{O_2} = -10{\cdot}3;\ Q_M = +2{\cdot}5;\ Q_M^{N_2} = +20{\cdot}3.$$

Table VI shows the figures obtained for the brain of guinea-pigs dying of rabies.

Table VI.

Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
− 9·2	+2·9	+25·6
− 8·8	+2·7	+18·8
− 7·0	+1·0	+20·4
−10·8	+1·9	+20·2

Here no hyperplasia is found and the activities of an intracellular virus seem to cause no alteration in the carbohydrate metabolism of the cells.

ROUS CHICKEN SARCOMA.

Warburg only quotes one experiment on the metabolism of this tissue, showing a behaviour similar to that of the rat tumours, studied so extensively.

Many measurements, in Ringer solution, have been made and Table VII shows the great variability in the results obtained. The respiration was, in some cases, found to be abnormally high, which had the effect of making U negative, and the Meyerhof quotient very low. In all cases a histological control showed no areas of necrosis, only the outer shell of the growth being used for the experiments. It has not been possible to correlate these wide variations with the age of the bird or the general necrotic or haemorrhagic conditions of the tumour.

Table VII.

No. of days after transmission with cell-free filtrate	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	U
10	− 8·7	+20·3	+27	0·8	+ 9·6
11	− 4·0	+13·8	+21·8	2·0	+13·8
13	−22·0	+23·9	+36·6	0·6	− 7·4
14	−26·9	+21·3	+30·1	0·3	−23·7
14	−10·1	+18·1	+28·1	1·0	+ 7·9
10	−20·0	+21·2	+33·3	0·6	− 6·7
10	−10·4	+16·3	+31·8	1·5	+11·0
11	− 7·2	+16·2	+29·2	1·8	+14·8
12	− 4·8	+22·6	+33·8	2·3	+24·2
13	− 7·2	+21·8	+35·5	1·9	+21·1

In order to study any possible immediate effect of the agent responsible for the initiation of Rous sarcoma on the metabolism of the muscle cells near the site of injection, a series of measurements was made at daily intervals from the time of transmission.

Cut muscle under the experimental conditions shows a low metabolism and a control experiment was made simultaneously with muscle from the opposite breast of the bird. A typical value obtained for cut muscle is:

$$Q_{O_2} = -2{\cdot}5;\ Q_M^{O_2} = +1{\cdot}2;\ Q_M^{N_2} = +2{\cdot}5.$$

A slight increase in metabolic activity of the muscle was noticed during the first two days after injection of cell-free filtrate (possibly due to leucocytic reaction) followed by a return to the normal low values of the undisturbed muscle. Only when a histological examination showed foci of tumour cells scattered through the muscle was any real increase found in the metabolism quotients. The figures obtained showed a rough correspondence in their magnitude to what might be theoretically calculated from the proportion of tumour tissue seen microscopically. It would seem correct to conclude that the tumour cells possess their characteristically high metabolic rate from the time of their appearance rather than that a progressive development of this metabolism takes place over a transitional period.

Summary and Discussion.

1. Using the technique developed by Warburg, the carbohydrate metabolism of a series of lesions associated with intracellular viruses has been examined.

2. The activities of the virus in fowl-pox lesions, vaccinia lesions in young chickens, and in human warts, are accompanied by epithelial hyperplasia and in these cases an active metabolism has been found, corresponding in type to that characteristic of malignant tissue.

Making allowances for non-reacting tissue in the sections examined, the magnitude of the respiration and aerobic and anaerobic glycolysis approximates to that found for tumours.

3. In the case of vaccinia lesions in rabbits, where little or no epithelial hyperplasia is evident, the lower quotients obtained are probably due to leucocytic invasion.

4. The brain of guinea-pigs dying of rabies provides an example of a tissue where a virus is active without stimulating the cells to abnormal division, and no deviation is found from the normal metabolism.

5. The metabolic activity of fowl-pox lesions shown graphically exhibits a rough parallelism in its magnitude to the state of development or regression of the lesion.

6. Great variability in the values for respiration in the Rous chicken sarcoma are recorded.

7. After the injection of Rous sarcoma cell-free filtrate in the muscle of fowls a slight rise in the metabolism was noticed during $4\frac{1}{2}$ hours, followed by a return to the normal values obtained for resting muscle.

During the subsequent development of the tumour in its early stages, the figures obtained indicate that Rous sarcoma cells, on their first appearance, assume the high metabolic activity characteristic of the fully grown tumour.

The general conclusion which may be drawn from these results would seem to be that the magnitude and relationships of the respiratory and glycolytic processes, found by Warburg to be characteristic of malignant tissues, are not specific for malignant tissues but are a common feature of pathological overgrowths.

The author's thanks are due to Prof. Warburg of Berlin-Dahlem for demonstrating his methods, and to Dr G. Marshall Findlay of the Imperial Cancer Research Fund for his ready help in obtaining material and conducting the animal experiments.

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CLXI. THE PURINOLYTIC ENZYMES OF THE LEECH (*HIRUDO MEDICINALIS*) AND THE FRESH-WATER MUSSEL (*ANODONTA*).

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(*Received August 13th, 1928.*)

Introduction.

Uric acid is in almost every case a stage or else the final product of purine metabolism within the living organism. Certain cases have, however, been reported where this stage appears not to enter. Thus Ulpiani and Cingaloni [1906] found that certain bacteria derived from poultry excreta degrade aminopurines in a different manner, involving the formation of guanidine; in this case, therefore, neither uric acid nor allantoin can be amongst the products of purinolysis.

Certain invertebrate forms are known [Przylecki, 1926], *e.g.* leeches and fresh-water mussels, amongst the final products of whose purine metabolism uric acid or allantoin cannot be found. In the case of leeches uric acid added to their tissues is not oxidised, whilst *Anodonta* is able quantitatively to destroy added uric acid. Two explanations of these phenomena might be given. One would be that the purine catabolism of these animals runs a course similar to that of Ulpiani and Cingaloni's bacteria, whilst the other would be that purine catabolism stops short at some stage prior to the appearance of uric acid. The first supposition would involve a fall in the purine-N content of the tissues after autolysis of over 20 %; in the second case the loss of purine-N due to autolysis should not exceed 20 % if both guanase and adenase are present, or some smaller figure if only one of these enzymes exists, and would be nil if neither of these ferments is possessed by these animals.

Experimental.

A. *Leeches.* These animals were first placed in concentrated sodium chloride solution, with the object of forcing them to disgorge any blood present in the alimentary canal, and were then washed, roughly dried with filter paper, and comminuted using either scissors or a mincing machine. Four portions of 50 g. each of the well-mixed mince were weighed out and placed in litre flasks, to which 500 cc. of water were added. Toluene was added to two flasks, which were left in a thermostat at 37° for about two weeks. Sulphuric acid to 5 %

was then added and the contents of the flasks were boiled under reflux for 4 hours, after which total- and purine-N were determined by the method of Krüger and Schittenhelm, as described in a previous paper [Truszkowski, 1926]. A portion of mince was also weighed out for the determination of dry content, being dried at 110° to constant weight. The contents of the remaining two flasks were hydrolysed immediately. The results are given in Table I, each value being the mean of two determinations. The dry content varies from 19·25 to 27·2 %, average 22·5 %, and the total-N content from 2·718 to 3·675 %, average 2·912 %. Considerable variations are found in purine-N, from 48·0 to 68·4 mg. per 100 g., mean 56·7 mg. per 100 g. After autolysis (15–17 days) the mean purine-N content falls to 47·8 mg. per 100 g. giving a mean loss of 16·7 %. This indicates deamination of both aminopurines present. The absence of uniformity of the results obtained is probably due to the incomplete elimination of blood from the alimentary tract.

Table I. *Total- and purine-N contents of leeches and fresh-water mussels before and after autolysis.*

				Before autolysis			After autolysis		
Material No.	% solid substance	% N live wt	% N dry wt	Purine-N mg. per 100 g. live wt	Purine-N mg. per 100 g. dry wt	Days autolysis	Purine-N mg. per 100 g. live wt	Purine-N mg. per 100 g. dry wt	% diminution in purine N
Leeches 1	21·15	2·718	12·85	68·4	323·6	17	63·0	280·2	7·9
2	19·25	2·344	12·18	48·0	249·1	15	36·3	189·2	24·4
3	27·2	3·675	13·53	53·8	197·7	16	44·2	161·8	17·8
Mean	22·5	2·912	12·85	56·7	256·8	—	47·8	210·4	16·7
Mussels 1	12·7	1·179	9·27	36·2	284·8	—	—	—	—
2	13·0	1·093	8·41	34·0	261·5	15	28·1	216·5	17·4
3	12·75	0·963	7·55	36·0	282·6	16	31·8	249·5	11·7
4	11·1	1·001	9·10	34·6	314·3	13	29·8	270·8	13·9
Mean	12·39	1·059	8·58	35·2	285·8	—	29·9	245·6	14·3

B. *Fresh-water mussels.* These were taken from clay-pits in the vicinity of Warsaw at various seasons of the year, from spring to autumn; all belonged to various sub-types of the genus *Anodonta*. After separation from the shell, they were allowed to drain for a few minutes, and passed through a mincing machine. The well-mixed mince was then treated as above for the determination of dry content, total- and purine-N. In the determination of purine-N, however, it was found that Krüger and Schittenhelm's method was not, without certain modifications, applicable. This was due to the extraordinarily high calcium content of these animals; thus in No. 2, the filtrate after acid hydrolysis contained 1·4 g. of calcium oxide, amounting to over 21 % of the dry weight of tissue taken and the residue from hydrolysis contained a further quantity of insoluble calcium sulphate. This calcium, whilst soluble in strongly acid solution, begins to separate as a flocculent precipitate at about p_H 4, precipitation being complete at p_H 8, at which p_H sodium hydrogen sulphite

and copper sulphate are added for the precipitation of purines, and accompanies the purines through all the subsequent stages of the determination. Its harmful influence is particularly noticeable in the dissolution of the purine-copper double salt by means of sodium hydrosulphide, when, even after adding comparatively very large quantities of the sulphide, copper is continually found in the filtrate. This effect appears to be due to preferential formation of calcium sulphide or hydrosulphide.

This source of error was eliminated by the addition of excess ammonium oxalate to the hydrolysate, after its neutralisation and feeble acidification with acetic acid.

The dry content of *Anodonta* (Table I) varies from 11·1 to 13·0 %, mean value 12·4 %. The nitrogen content varies from 0·963 to 1·179 %, mean value 1·059 %, and the purine-N content from 34 to 36 mg. per 100 g., mean 35·2 mg. per 100 g. After autolysis (13–16) days the mean purine-N content fell to 29·9 mg. per 100 g., a decrease of 14·3 %. It appears therefore that in this case, too, both guanase and adenase are present.

Discussion.

Since both leeches and fresh-water mussels are capable of deaminising guanine and adenine, but do not produce uric acid, it follows that in these animals, purine catabolism stops at some stage short of uric acid. That these animals do not degrade purines in some other way not involving deamination and the production of uric acid is indicated by the limited loss of purine-N during autolysis.

It hence follows that the end-product of purine catabolism of leeches and *Anodonta* must be xanthine and/or hypoxanthine. Przylecki's classification [1926] of leeches as uricostatic-uriconegative forms and of *Anodonta* as uricolytic-uriconegative forms is thus confirmed.

Summary.

1. Purine-N has been determined before and after autolysis of the tissues of leeches and of fresh-water mussels.

2. The results obtained indicate that the end-product of their purine catabolism is xanthine and/or hypoxanthine.

This research was undertaken at the suggestion of Prof. S. J. Przylecki, to whom I wish to express my sincere gratitude for his help and advice.

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CLXII. STRUCTURE AND ENZYME REACTIONS.

PART VII. THE SYSTEM GLYCOGEN-AMYLASE-LIVER TISSUE.

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The researches described in the previous parts of this series [Przylecki *et al.*, 1927, 1928; Truszkowski, 1928], although performed upon models under conditions differing greatly from those obtaining within the living cell, yielded results possessing a certain biological importance, notably in the elucidation of the rôle of structure in the attainment of definite intracellular equilibrium.

It has been shown that the introduction of certain natural cell-constituents into the system amylase-glycogen leads to a retardation of the velocity of the reaction, due to the adsorption of either the ferment, the substrate, or both. We have, further, found that amylase becomes inactivated by adsorption upon a lipoid mixture, whilst adsorption upon protein in no way impairs its activity. It appears, therefore, that the velocity of glycogenolysis in the presence of these cell-constituents depends not only upon that of the ferment.

One of the problems whose solution was sought in this series of researches was that of the domination within the cell of reactions of synthesis over those of hydrolysis. On the basis of the experiments described in Parts v and vi of this series it was concluded that, in view of the strong adsorption of glycogen upon cell-proteins, and of the feeble adsorption of glucose, the reaction glucose $\rightleftharpoons$ polysaccharides should be predominantly one of synthesis in a cellular environment. The conditions under which the experiments on which this conclusion was based are, however, not entirely comparable with those obtaining within the cell.

It was therefore necessary to study this problem under conditions closer to those actually found within the cell, not only in so far as concerns the nature of the adsorbent itself, but also in order to establish whether changes in the equilibrium point in favour of the reaction of synthesis of glycogen are due exclusively to adsorption of both constituents, or whether some further factors come into play, as indeed appeared to us to be probable.

In view of the high degree of adsorption of amylase found by us, and of the efficient action of such adsorbed amylase, the equilibrium point of the reaction

glucose $\rightleftharpoons$ glycogen, in the system glycogen-glucose-amylase-protein is, as we have shown, dependent upon the ratio of unadsorbed glycogen to glucose. The degree of glycogenolysis depends, therefore, upon the reversibility of the adsorption of glycogen.

The first question to be settled was whether glycogen in adsorption upon protein can be eluted by addition of water, and experiment showed that this was the case. In view of this, equilibrium in this system would depend upon the two equilibrium relations:

$$\text{adsorbed glycogen} \rightleftharpoons \text{free glycogen (I)};$$

$$\text{free glycogen} \rightleftharpoons \text{glucose (II)}.$$

The elution of glycogen from protein would proceed until definite equilibrium had been attained in the system free glycogen-glucose, and this equilibrium again would depend upon the ratio adsorbed glycogen : free glycogen. Thus the greater the percentage of glycogen in adsorption, the sooner would equilibrium II supervene, and the quantity of adsorbed glycogen would be greater, the greater the ratio of free glycogen to glucose. In view of the above, we should expect to obtain minimum glycogenolysis in the presence of active amylase under the following conditions.

1. In order to bring the ratio of adsorbed to free glycogen to a maximum, very large quantities of protein together with small concentrations of glycogen should be taken; that is, however, inconsistent with biological conditions.

2. In order to attain an optimum ratio of free glycogen to glucose, solutions containing high concentrations of glucose and colloids should be taken. The latter point appeared to us to be of special importance.

The first question examined experimentally therefore was whether the deposition of glycogen in liver cells could be explained as being due exclusively, or to a large extent, to adsorption on the surfaces of the various hepatic colloids. This appeared to us to be *a priori* somewhat improbable, but, nevertheless adsorption isotherms of glycogen on liver pulp were constructed. The liver pulp used was left for a considerable period of time with antiseptics, which were then removed, and the pulp was thoroughly washed. The product contained no amylase or polysaccharides, and was also partly freed from lipoid substances. The reversibility of adsorption of glycogen on this pulp was next studied, and finally, having settled this point, the equilibrium point of the reaction glycogen $\rightleftharpoons$ glucose was determined in the system adsorbed amylase-glycogen-liver protein, using large quantities of the last-named substance.

Experimental.

I. *Adsorption.*

The degree of adsorption on liver pulp of glycogen from solutions of various concentrations was determined according to the methods described in Part III of this series [1928]. The liver pulp was prepared by adding to 1 kg. of comminuted, and, as far as possible, blood-free liver, 100 cc. of chloroform, 200 cc.

of glycerol and 1 litre of water. The whole was left for some months, during which time amylase and glycogen passed into solution, and the latter underwent hydrolysis to glucose. The contents of the flask were then poured through a sieve, on which the residue was washed in a strong current of water for 48 hours. The resulting product, which was free from amylase and polysaccharides, was allowed to drain, and was then used as the adsorbent. To 10, 20, or 30 g. portions of this pulp 30 cc. of 0·5–10 % glycogen solution were added, and the whole was shaken for 4 hours. The mixture was then either filtered, or centrifuged at low velocities in order to avoid concentration of glycogen in the lower layers. Glycogen was determined either in the filtrate or in the pulp, using the Pflüger-Bertrand method.

Table I. *Adsorption of glycogen on liver pulp.*

In every case 30 cc. of glycogen solution were used, and the pulp was rubbed through a sieve before weighing out.

Glycogen solution used		Adsorption of glycogen on: 10 g. pulp (analysis of pulp)		20 g. pulp (analysis of solution) Exp. 1			20 g. pulp (analysis of solution) Exp. 2			30 g. pulp (analysis of pulp)		
						Free glycogen in solution			Free glycogen in solution			Free glycogen in solution
%	mg.	%	mg.	%	mg.	%	%	mg.	%	%	mg.	%
0·5	150	53	79·5	96	144	0·018	82	123	0·088	—	—	—
1·0	300	52	156	94	282	0·054	85	255	0·179	99·0	297	0·099
2·0	600	46	276	66·6	400	0·60	79	474	0·416	—	—	—
3·0	900	43	387	68·8	619	0·87	70	630	0·891	93·0	837	0·21
5·0	1500	40	600	—	—	—	72	1080	1·39	—	—	—
7·5	2250	—	—	76·0	1710	1·62	—	—	—	—	—	—
10·0	3000	—	—	78·1	2343	1·97	74	2220	2·57	89·1	2673	1·08

The results, given in Table I, show that the pulp has an extraordinarily high adsorptive power for glycogen, and that, using appropriate concentrations of pulp, corresponding to those present in the liver, this adsorption may, even for 10 % glycogen solutions, amount to as much as 90 %. This fact appears to be of great interest, as it indicates that, within the cell, glycogen is concentrated on the protein surfaces. These results are, moreover, in conflict with certain histological evidence, whereby it would appear that glycogen is concentrated on lipoid surfaces. We cannot yet say whether the inability to adsorb glycogen found by Truszkowski for certain lipoids applies to all lipoids, or whether the drops of lipoid substances within the cell are coated with some protein, on the surface of which glycogen may be adsorbed. It is also possible that diminution of the surface of drops of lipoids due to several drops coalescing into one large one may lead to the irreversible precipitation of glycogen or starch adsorbed on these surfaces. This question will be considered in a later paper.

It follows from the results described in this paper that, where a cell system contains up to 10 % of glycogen, barely 0·8 % or even less can exist in solution, the remainder being adsorbed upon protein and possibly lipoid surfaces.

II. *Reversibility of adsorption.*

Three points of biological significance were here to be elucidated. The first was to establish with what facility glycogen undergoes elution from protein as a result of dilution. The second was to determine the extent of adsorption of glycogen from solutions of various concentrations. Finally, the percentage of glycogen remaining in solution after the addition of different volumes of the same glycogen solution to equal quantities of adsorbent was measured.

The preliminary experiments were carried out on liver pulp or coagulated egg-white on which glycogen was adsorbed. Analysis of the adsorbent after washing in a stream of water for 24–48 hours showed that barely traces of glycogen remained. It hence follows that glycogen can be quantitatively eluted from adsorption on protein without the agency of elutive factors other than water. Further experiments were of a quantitative nature, the percentage elution at given dilutions being determined. The results are given in Table II.

Table II. *Elution by dilution.*

In every case 20 g. of liver pulp were taken.
In Exp. 4 30 cc. of 5 % glycogen were added to 20 g. of pulp, the whole was shaken for 4 hours, left for 3 hours, 270 cc. water were then added, the whole was shaken a further 6 hours and left 12 hours, after which glycogen was determined.

No. of Exp.	Glycogen g.	Water cc.	Glycogen %	Adsorbed glycogen		Glycogen in solution	
				g.	%	g.	%
1	0·150	30	0·5	0·123	82	0·027	0·088
2	1·500	30	5·0	1·170	78	0·330	1·09
3	1·500	300	0·5	0·688	46·5	0·812	0·27
4	1·500	300	0·5	0·741	49·4	0·759	0·25

III. *Definitive equilibrium.*

The following experiments have only a theoretical significance, their object being to establish whether in the presence of an adsorbent, definitive equilibrium supervenes before the hydrolysis of all the glycogen present in a system containing on the average 70–80 % of water.

These experiments were performed on solutions of glycogen, the adsorbent being coagulated egg-white, on which amylase had been adsorbed. 10 g. of egg-white with adsorbed amylase were taken, and 5 cc. of solutions or suspensions of 1 to 10 % glycogen. These systems were kept under aseptic conditions, without the introduction of antiseptics. In order to favour the reaction glucose → glycogen, glucose was added to 1–2 % of the reaction solution. The glycogen content of the whole system was determined after 1, 2 and 4 weeks. The results indicated that glycogenolysis proceeds practically to completion in these circumstances.

As to the cell system, the above experiments indicate that adsorption is able to inhibit glycogenolysis and to keep glycogen stabilised for an indefinitely long period only when glycogen is present exclusively in adsorption, or when

the reaction glycogen $\rightleftharpoons$ glucose is close to equilibrium, so that it is able to proceed in one direction or another when changes supervene in the concentration of one or the other substrate. Only in these circumstances can the synthesis of polysaccharides take place.

For the realisation of this synthesis not only the presence of an adsorbent which would remove large quantities of glycogen from solution is necessary, but, further, conditions should be such that the reaction glycogen $\rightleftharpoons$ glucose should be easily reversible. Such conditions cannot be brought about by the presence of an adsorbent. For this purpose, not only should the relative concentration of glycogen be diminished but also that of water. This matter will be considered more fully in a subsequent communication.

A further conclusion may be drawn from the above results. This is that our hypothesis is confirmed that the velocity of reaction is, in view of the traces of glycogen left in solution, determined in the first place by the equilibrium adsorbed glycogen $\rightleftharpoons$ free glycogen, even where the concentration of polysaccharide is high.

SUMMARY.

1. Liver tissue has an exceptionally high adsorptive action upon glycogen.

2. Adsorption of glycogen upon tissue and other proteins is of a reversible nature, elution taking place when the concentration of glycogen in solution falls.

3. The addition of protein to the system glycogen-amylase retards but does not inhibit the reaction of glycogenolysis.

4. Biologically, glycogenolysis is the resultant of the reactions: adsorbed glycogen $\rightleftharpoons$ free glycogen $\rightleftharpoons$ glucose.

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CLXIII. DECOMPOSITION OF URIC ACID IN BLOOD.

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(*Received August 27th, 1928.*)

Garot [1926] showed that the uric acid content of dog's blood falls considerably after digestion with "pancreatin"; this effect he ascribed to the presence of uricase in the blood of these animals. Flatow [1926] found that uric acid undergoes oxidation if added to blood, and that this process is greatly accelerated by passing air through the mixture. The uricolytic agent in this case was, according to this author, a substance which he termed "uricoid."

Up to the present, no incontrovertible evidence has been adduced for the existence of uricase in the blood of any mammal, and the nature of the substance producing uricolysis is therefore still problematical.

Biltz and Schauder [1923] and Piaux [1924] showed that a number of substances exist which catalyse the oxidation of uric acid, the most important being such metals as iron, copper, manganese. Since an iron-containing substance, haemoglobin, is present in blood, the possibility arose that this was the factor responsible for the oxidation of uric acid in blood.

Experiments will be described in this paper having as their object the elucidation of the nature of the catalyst in question and of the action of haemoglobin upon uric acid.

Experimental.

A. *Determination of uricolytic agent.*

Horse blood was collected at slaughter-houses, and potassium oxalate added immediately to prevent clotting. It was used generally within about 6 hours of shedding.

The uricolytic powers of whole blood, blood-plasma, erythrocytes, boiled erythrocytes, stromata and haemolysed corpuscles free from stromata were determined separately. Two types of determination were made. In one, uricolysis was determined in bottles through which a feeble stream of oxygen was passed, whilst in the other determinations were made in stoppered flasks. In all cases 50 cc. of plasma, erythrocyte suspension, etc., were taken, and 20 cc. of lithium urate solution together with 20 cc. of water were added,

with thymol as disinfectant. The reaction mixtures were placed in a thermostat at 37°, and their uric acid content determined at different intervals of time.

The solution was freed from protein by the method of Folin and Wu, 100 cc. of 10 % sodium tungstate and 100 cc. of 2/3 *N* sulphuric acid being added. Uric acid was determined in the filtrate using the colorimetric Folin-Denis method, 12 cc. of filtrate being taken for each determination. The results obtained by this colorimetric method were confirmed by determination of purine-nitrogen using the method of Krüger and Schittenhelm.

The results given in Table I show that blood-plasma possesses no uricolytic power, since even after 48 hours the uric acid content did not diminish by more than 6·8 %, and this diminution may be ascribed to adsorption. In the presence of erythrocytes uric acid undergoes oxidation. An examination of the figures obtained shows that at first adsorption takes place; during the first two hours no difference in the content of uric acid can be observed, after which it undergoes continual destruction, so that after 48 hours about 73 % is oxidised.

In order to establish whether the agent responsible for this oxidation is a catalyst or a ferment, the above experiments were repeated with erythrocytes previously heated to 100° for 10 minutes. The results (Table I) show that their uricolytic power is in no way diminished by this treatment, whence it follows that blood contains a thermostable uricolytic agent.

Whilst crude uricase is inactivated by such treatment, it has been shown by Przylecki (unpublished work) that highly purified preparations of uricase are similarly thermostable. In order to verify whether this ferment is present, the procedure employed by Przylecki was applied to the separation of uricase. The product so obtained was inactive, whence it would appear that uricase is absent from mammalian blood.

The effect upon velocity of oxidation of passing a feeble stream of oxygen through the reaction mixture was next studied. The results obtained (cf. Table I) showed a distinct acceleration of reaction, since only traces of uric acid remained after 48 hours.

We have, therefore, established that erythrocytes contain some thermostable non-enzymic substance which energetically oxidises uric acid in the presence of oxygen. The question remains whether this substance is present in the stromata or the contents of the erythrocytes. Accordingly, a suspension of haemolysed erythrocytes was centrifuged, the supernatant liquid passed through a Berkefeld filter, in order to ensure the removal of fragments of stromata, and uric acid added to the filtrate. Only traces of uric acid remained after 48 hours (Table I). This result was confirmed by determining purine-nitrogen using the method of Krüger and Schittenhelm. The purine-nitrogen content fell in 48 hours from 25·5 mg. to 4·9 mg. (see Table III). Parallel experiments on washed stromata showed only absorption of uric acid, amounting after 48 hours to 10·5 %, after which no further fall in uric acid content could be observed (Table I).

Table I.

Catalyst	Original uric acid content mg. in 2 cc.	5 mins. Uric acid content mg.	30 mins. Uric acid content mg.	30 mins. Loss %	1 hour and 2 hours. Uric acid content mg.	4 hours Uric acid content mg.	4 hours Loss %	24 hours Uric acid content mg.	24 hours Loss %	48 hours Uric acid content mg.	48 hours Loss %	72 hours Uric acid content mg.
Plasma	0·852	No change	0·795	6·68	No change	No change		No change		No change		No change
	0·852	,,	0·793	6·92	,,	,,		,,		,,		,,
Mean	0·852	—	0·794	6·8								
Fresh erythrocytes ...	1·165	No change	1·107	4·97	No change	1·019	12·55	0·852	26·86	0·333	71·41	
	1·186	,,	1·107	6·66	,,	1·019	14·08	0·862	27·31	0·299	75·21	
	1·126	,,	1·048	6·92	,,	0·96	14·2	0·843	25·13	0·303	73·08	
Mean	1·159	—	1·087	6·18	—	0·999	13·64	0·852	26·43	0·311	72·23	
Boiled erythrocytes ...	1·165	No change	1·107	4·97	No change	1·019	12·53	0·803	31·07	0·320	71·84	
	1·186	,,	1·107	6·66	,,	1·019	14·08	0·941	20·66	0·392	68·83	
	1·126	,,	1·107	1·68	,,	0·862	23·52	0·666	40·9	0·284	74·8	
Mean	1·159	—	1·107	4·47	—	0·966	16·71	0·803	30·87	0·334	71·82	
Erythrocytes with oxygen	1·153	—	—	—	—	—	—	0·367	68·17	Traces		
	1·127	—	—	—	—	0·896	22·28	0·372	66·99	,,		
						0·96	14·81	0·369	67·58	,,		
Mean	1·14	—	—	—	—	0·928	18·54					
Intracorpuscular fluid ...	1·15	—	—	—	—	—	—	—	—	Traces		
	1·127	—	—	—	—	—	—	—	—	,,		
Stroma 0·8 g. per portion	0·55	—	—	—	—	—	—	0·534	2·9			
	0·372	—	—	—	—	—	—	0·361	3·06	0·495	10	No change
										0·331	11·02	
Mean	0·461	—	—	—	—	—	—	—	2·98	0·463	10·51	No change
Haematin 0·5 g. per portion	1·14	—	—	—	—	—	—	—	—	Traces		
	1·127	—	—	—	—	—	—	—	—	,,		

The uricolytic agent is therefore present exclusively in the intracellular fluid, and it appears probable that it may be haemoglobin. This conclusion is further supported by experiments conducted on purified crystalline haematin, in the presence of which uric acid was similarly quantitatively oxidised.

B. *Influence of alcohols, of methylene blue and of potassium cyanide upon the oxidation of uric acid in blood.*

Butyl and propyl alcohols were added in various concentrations to solutions of haemolysed blood containing uric acid. The results, given in Table II, show that oxidation is inhibited to an extent depending upon the concentration of alcohol added.

In order to establish whether we have here to do with a reaction of true oxidation or of dehydrogenation, the influence upon the velocity of oxidation of the addition of methylene blue was investigated. The results (Table II) do not indicate any acceleration of reaction where this dye is added; on the contrary, a slight retardation is observed, possibly due to adsorption of methylene blue upon the surface of the catalyst in question.

The addition of potassium cyanide did not inhibit uricolysis (Table II).

Table II.

Substance added cc. per 100 cc. erythrocyte suspension	Original uric acid content mg./5 cc.	After 48 hours	
		Uric acid content mg./5 cc.	% loss of uric acid
	1·15	Traces	
Butyl alcohol 2	1·15	0·16	86·95
Butyl alcohol 4	1·15	0·605	47·3
Propyl alcohol 8	1·15	0·63	45·12
Propyl alcohol 16	1·15	0·785	31·73
M/500 methylene blue 10	0·372	0·145	61·02
	0·55	0·2	64·05
Mean	0·461	0·1725	62·76
M/1000 potassium cyanide 40	0·53	0·14	73·54
	0·64	0·16	75
Mean	0·58	0·15	74·27

C. *The products of oxidation of uric acid in blood.*

The following analyses were carried out with the object of identifying the products arising from the catalytic oxidation of uric acid under the influence of haemoglobin. Urea and ammonia were determined by the methods of Folin and Wu in fresh blood, and in blood to which uric acid had been added and which had remained for 48 hours at 37°. Allantoin was determined in other portions, using Wiechowski's method, and in separate portions purine-nitrogen was determined by the method of Krüger and Schittenhelm. The results, given in Table III, show that the major portion of the uric acid-nitrogen is found as urea, the remainder being ammonia-, allantoin- and purine-nitrogen.

Table III.

	Fresh erythrocytes mg. N in 100 cc.	After 48 hours mg. N in 100 cc.
Total uric acid content of blood	25·5	4·9
Ammonia	—	2·4
Urea	4·2	12·6
Allantoin	—	6·3
Totals	29·7	26·2

Discussion.

Uric acid, on standing with blood, is quantitatively oxidised within 48 hours, yielding as products of oxidation, allantoin, urea and ammonia. The catalytic agent in question is absent from the blood-plasma and from the stromata of the erythrocytes, which are able only to adsorb uric acid in quantities not exceeding 10 % of that present in solution. Haemoglobin is probably the uricolytic catalyst in question.

It is well known that iron compounds play an important rôle as oxidation catalysts within the cell [see Warburg, 1914].

Robinson [1924], who used haemoglobin and haematin as catalysts for the oxidation of linseed oil, found that haematoporphyrin was without effect, and her experiments suggest that it is the iron present in haemoglobin that is responsible for oxidation of uric acid. This author showed further that cyanides do not inhibit the catalytic action of iron present in haemoglobin. This would point to the iron being present in some non-ionised form, as is the case with that of haemoglobin.

The results obtained with methylene blue indicate that the reaction of uricolysis under the influence of haemoglobin is rather one of oxidation than one of dehydrogenation.

Summary.

1. Uric acid is quantitatively oxidised by blood within 48 hours at 37°.
2. Stromata and blood-plasma are inactive in this respect.
3. Haemoglobin is most probably the uricolytic agent present in blood.
4. The products of uricolysis in blood are allantoin, urea and ammonia.
5. The reaction of uricolysis is inhibited by propyl and butyl alcohols, but not by cyanides.
6. The reaction is one of true oxidation, and not of dehydrogenation.

The above research was undertaken at the suggestion of Prof. S. J. Przylecki, to whom I wish to express my sincere gratitude for his assistance during its prosecution.

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CLXIV. A NOTE ON VOLATILE SULPHIDE FROM MUSCLE.

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(*Received August 21st, 1928.*)

The leg muscles of a well-nourished, recently killed guinea-pig were cut into small pieces by scissors and boiled in a distillation flask containing water, the delivery tube of which dipped below the surface of lead acetate solution. If the muscle was from a well-fed, sleek animal and was boiled immediately after the killing no volatile sulphide could be detected.

When some hours, preferably twenty-four, elapsed between killing the well-nourished animal and removal of the muscles, the presence of sulphide in the distillate was obvious. To remove the possibility of bacterial decomposition various procedures were adopted. In some the animals when killed were immediately eviscerated and the abdominal cavity was swabbed out with 1 % mercuric chloride solution. In others the amputated lower limbs were suspended, with skin intact or removed, in air containing toluene vapour. The results were the same in all cases. Apparently, therefore, autolysis makes a change in the muscle proteins whereby loosely bound sulphur is produced or liberated.

When the muscle was taken immediately after killing from a guinea-pig starved for 48 hours sulphide was readily detectable in the steam-distillate. These experiments were repeated many times and always with the same results. It is probable that the muscle of different animals shows considerable variation in this reaction. The flesh of sheep in poor condition emits sulphide readily on boiling but I have not succeeded in getting this with beef.

CLXV. ON THE OXIDATION OF CHOLESTEROL BY MOLECULAR OXYGEN.

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(*Received August 18th, 1928.*)

I.

Whilst the ability of the animal organism to synthesise cholesterol seems to be clearly proven, the question if, or to what extent, the cholesterol is broken down in oxidative or other ways in the body is still very obscure. This question is, indeed, not only of physiological interest, but in view of the diseases which are known to be accompanied by an abnormal accumulation of cholesterol in blood or tissues it is also of a definite pathological-clinical interest.

What is known about this question we owe to a great extent to Lifschütz [1927]. According to this author cholesterol is transformed by the action of permanganate or, better, benzoyl peroxide in acetic acid solution into a resinous product called "oxycholesterol." This substance can be recognised and estimated with the aid of a colour reaction with glacial acetic acid and sulphuric acid not given by the cholesterol itself. Since oxycholesterol has not been obtained crystalline and has no definite melting-point, the purity of the material is not yet assured. Lifschütz ascribes to it the formula $C_{26}H_{47}O_2$.

Oxycholesterol has been found by Lifschütz (and others) to be a normal constituent of the blood. It also seems to be present in certain other tissues. Lifschütz regards it as the first step in the break-down of cholesterol in the body. In spite of its indefinite chemical nature it merits, nevertheless, due attention, especially as a possible normal catabolite of cholesterol.

When protected from light pure cholesterol kept at room temperature seems to be very stable against the oxygen of the air. In the dry state it remains under such circumstances unchanged at least for weeks. Suspended in water it can be aerated for hours at 90–100° without perceptible changes (see below). Aeration of an alcoholic solution at 60–70° for a few hours at any rate (as we have found) does not affect it.

The mechanism by which the oxycholesterol is produced in the organism is not known. Lifschütz [1914] has found that oxycholesterol is formed when pure cholesterol in glacial acetic acid is heated for some days together with small amounts of dried, fat-free blood. He therefore is of the opinion that the primary oxidative attack on the cholesterol takes place in the blood. More recently Robertson [1925], in the course of experiments on the multiplication

of infusoria, found that cholesterol suspended in water in the presence of mere traces of substances from the acetone extract of brain tissue, when aerated at 100°, was oxidised to oxycholesterol. Perfectly pure cholesterol did not undergo oxidation under these conditions.

These observations of Robertson seemed to us to form a possible starting-point in the study of the mechanism by which the cholesterol is physiologically broken down. When we were already engaged in our work a paper was published by Moore and Willimott [1927] bearing on this subject. These authors found that when pure cholesterol in open dishes was heated to a temperature slightly above the melting-point it acquired after a few minutes a faintly yellow tint and afterwards gave a positive oxycholesterol reaction; they were not able to confirm Robertson's results that pure cholesterol suspended in water was stable when aerated at 90–100°. In such instances they also obtained invariably a positive oxycholesterol reaction. Moreover, pure suspensions without aeration directly evaporated at 100° gave similar positive tests. On the other hand the residues obtained after aeration for 48 hours at 37° with subsequent evaporation at the same temperature gave no coloration with the Lifschütz reagent. On account of these findings Moore and Willimott seem to be of the opinion that in the cases mentioned formation of resinous products with chromogenic properties similar to those of oxycholesterol is not due to an oxidation by oxygen but is an effect merely of heat. They recommend caution "in accepting the production of oxycholesterol as a specific effect of some particular mechanism in cases where heat is also involved."

That in the first of the above-mentioned observations the formation of oxycholesterol is due not merely to heat but also to the action of oxygen is, however, easily shown.

Two portions of pure cholesterol weighing each about 0·04 g. were heated at 150–155° for 20 minutes. Portion 1 was lying on an open watch-glass, portion 2 was placed in a small flask through which was led a very slow stream of pure nitrogen. After solidifying, portion 1 had a faintly yellow colour, was semi-transparent, and gave an intensively positive reaction for oxycholesterol. Portion 2 remained perfectly white and opaque and gave no trace of a positive Lifschütz reaction. When heated to about 155° in oxygen instead of in air the cholesterol acquired a yellow tint in a few minutes and showed a strongly positive oxycholesterol reaction. It was also found that if cholesterol suspensions, which, either "pure" or with an activating agent added, on aeration at 100° gave oxycholesterol, were treated with nitrogen at the same temperature, no oxycholesterol was formed.

The indispensable rôle of oxygen for the formation of oxycholesterol in these cases seems to be thus quite clear. The question next arises regarding the physical or chemical conditions necessary for the oxidation of cholesterol suspended in water by molecular oxygen. The following investigations are to be considered mainly as an inquiry into this question. Our experiments have been restricted to simple chemical systems.

II.

At first our experience of the formation of oxycholesterol in "pure" suspensions was similar to that of Moore and Willimott. We had at our disposal a sample of cholesterol prepared in this institute from human gall stones. The preparation was perfectly white and gave no reaction for oxycholesterol. The melting-point was not determined. 0·5 and 1 % suspensions of this cholesterol were prepared by dissolving the substance in a little alcohol and pouring the solution with stirring into water heated to 60–70°. The suspensions were aerated at 90–100° with and without addition of acetone extract from brain tissue for 30 minutes up to 5 hours. No consistent results were obtained. In most cases the residues gave a strongly positive reaction for oxycholesterol. Occasionally, however, negative reactions were obtained, also in cases where brain extract had been added. In order to get reproducible results we tried to standardise as far as possible the experimental conditions, and to avoid impurities from different sources. Accordingly we submitted the cholesterol to purification by recrystallising it seven times from alcohol. Thereafter it was no longer possible to get suspensions of the same strength as before, since most of the cholesterol flocculated immediately. In order to obtain as concentrated suspensions as possible we followed the directions given by Svedberg [1924], who emphasises the importance of boiling off the solvent completely to obtain stable colloids. Accordingly, the alcoholic solution of the cholesterol was ejected in small portions into boiling water through the end of a glass tube of fine bore. Most of the alcohol thereby immediately volatilised. The rest was driven off by distilling the fluid to half the bulk, but we did not succeed in obtaining more concentrated suspensions than could be obtained by simply pouring the alcoholic solution with stirring into hot water. The highest concentrations attained were 0·07–0·08 %.

Even after the measures mentioned oxycholesterol was formed on aeration of "pure" cholesterol suspensions at 90–100°. The results, although less irregular than in the first series of experiments, were, however, quantitatively not reproducible. The cholesterol was then further purified in different ways: (1) by bromination and subsequent reduction with zinc dust, (2) by boiling for one hour with 0·5 *N* alcoholic potassium hydroxide, diluting with water, extracting with ether and taking up the residue after evaporation of the ether in light petroleum, (3) by boiling an alcoholic solution of cholesterol twice with blood charcoal (Merck's pure) for half an hour. After these purifications the cholesterol was once recrystallised from alcohol and then aerated at 90° for one hour. In all instances the Lifschütz reaction of the residue was positive. Considering the possibility that traces of oxycholesterol (or decomposition products of it) might favour its production by autocatalysis the residues obtained by evaporating the mother-liquors from the above-mentioned recrystallisations were tested for oxycholesterol. They all gave a positive reaction. The cholesterol purified with charcoal was then further recrystallised from

alcohol (4 times) until the residue from the mother-liquor no longer gave any reaction for oxycholesterol. The suspensions made with cholesterol thus prepared could be aerated for several hours (up to 7) without production of oxycholesterol in demonstrable amounts or, sometimes, with the formation of oxycholesterol in amounts giving only a faintly positive reaction. The method of purification at last adopted was the following. The crude cholesterol from gall stones was, after four recrystallisations from alcohol, boiled twice with blood charcoal in alcoholic solution for half an hour. The cholesterol was then further recrystallised from alcohol (3 or 4 times) until the residue from the mother-liquor no longer gave any positive test for oxycholesterol. The preparation thus obtained melted at 148° (corr.). Whether oxycholesterol actually acts autocatalytically was not further investigated. All that can be said is that the mode of purification above described proved to be reliable in repeated experiments for our purpose[1]. The highest concentrations of the suspensions prepared from this cholesterol were from 0·05 to 0·06 %.

III.

In the course of this investigation we made an observation which seemed to us to be worth closer study. If merely a trace of an alkali soap was added to the cholesterol suspension we found regularly an abundance of oxycholesterol on aeration at 90°.

Experimental.

(1) The suspensions were prepared either according to Svedberg or simply by dissolving 0·05 g. cholesterol in 10 cc. alcohol and pouring the solution with stirring into 100 cc. water at 60–70°. In the earlier experiments we distilled off all the alcohol, but later found that this was not necessary. In some instances the long boiling necessary to drive off the alcohol seemed to make the suspension more prone to the formation of oxycholesterol, but with suspensions prepared in this way we obtained residues giving no reaction for oxycholesterol after aeration at 90° up to 7 hours.

(2) For the aeration 20 cc. of the suspensions were placed in a wide test-tube closed with a rubber stopper provided with three holes, respectively for inlet and outlet of air and for a thermometer. The glass tube for the inlet of air was in its lower submerged end enlarged to a bulb perforated with small holes. The outlet tube was connected with a reflux condenser in its turn connected with a suction pump. The air was in most experiments purified by filtering through cotton wool and by passing through three wash-bottles containing strong alkali, concentrated sulphuric acid and water respectively. The test-tube was immersed in a water-bath heated to the desired temperature. When the air was sucked through the suspensions in the boiling water-bath

[1] It was found that if the cholesterol thus purified was again boiled with charcoal the residue from the mother-liquor in the first recrystallisation from alcohol gave a positive reaction. Obviously small amounts of oxycholesterol are formed by the boiling with charcoal.

the temperature of the former sank, of course, below 100°, the more rapid the air stream, the lower the temperature. The temperature of the suspension therefore served as a relative measure of the rate of the air stream. By regulating the suction pump so that the temperature of the suspension was constantly at 90° we had a simple means of getting approximately the same rate of air stream in the different experiments at this temperature.

(3) The evaporation of the suspensions was performed in porcelain dishes placed in an air stream at room temperature. As the soaps interfere with the Lifschütz reaction the residues were extracted with light petroleum (B.P. 35–55°) and the latter was thereafter driven off. All vessels used were carefully cleansed with hot sulphuric acid-dichromate mixture.

(4) In some experiments estimation of the oxycholesterol was made roughly by direct inspection of the intensity of the colour reaction, in others by spectrophotometrical determination. A sample of oxycholesterol, prepared according to Lifschütz by oxidation of cholesterol by benzoyl peroxide, served as a standard. To 1 cc. of a chloroform solution of oxycholesterol was added 2 cc. of the reagent. Instead of adding, as Lifschütz did, the ferric chloride afterwards, we dissolved this substance in a concentration of 1/1000 directly in the reagent (9 vol. glacial acetic acid and 1 vol. conc. sulphuric acid). The spectrophotometrical reading was made after the mixture had been left for 15 minutes at room temperature. The spectral range used was that between 625 and 645$\mu\mu$. The relation between the concentration of the oxycholesterol and the extinction coefficient was determined in a series of oxycholesterol solutions of varying concentrations. The spectrophotometer used was one of Hüfner's type.

The sodium stearate and sodium oleate used were pure preparations from Merck, the rest of the soaps tried were prepared by adding the calculated amount of alkali to the free fatty acids (Kahlbaum's). Table I shows the amount of oxycholesterol formed on addition of sodium stearate in varying concentrations. The suspensions were aerated for one hour at 90°. Repeated control experiments (without soap) at 90° and lower temperatures gave negative results. The amount of oxycholesterol formed is given as a percentage of the amount of cholesterol taken (the difference in the molecular weights referable to the oxygen uptake not being taken into consideration).

Table I.

Stearate concentration %	Oxycholesterol formed %
0·02	71
0·02	52
0·004	45
0·004	65
0·001	49
0·001	42
0·0002	5
0·0002	0
0·0002	0

Table II shows the production of oxycholesterol on aeration at different temperatures.

Table II.

A. Stearate concentration 0·004 %			B. Stearate concentration 0·02 %		
Aeration (hours)	Temperature	Oxycholesterol formed %	Aeration (hours)	Temperature	Lifschütz reaction
1	90°	65	1	90°	Strongly positive
1	85°	65	1	70°	Negative
1	80°	40	2	70°	Positive
1	75°	25	4	70°	Strongly positive
1	70°	10	1	60°	Negative
1	65°	0	3	60°	Faintly positive
1	65°	0	6	60°	Strongly positive
			3	55°	Negative
			6	55°	„
			12	55°	Positive
			12	45°	Negative

Tables III and IV show series of similar experiments with addition of sodium oleate. The controls were negative.

Table III.

Aeration for 1 hour at 90°.

Oleate concentration %	Oxycholesterol formed %
0·02	57
0·02	57
0·02	98
0·004	25
0·004	14
0·004	25
0·001	0
0·001	13
0·001	10
0·0002	0
0·0002	0

Table IV.

Oleate concentration 0·02 %.

Aeration (hours)	Temperature	Lifschütz reaction
1	90°	Strongly positive
1	80°	Positive
3	80°	Strongly positive
1	70°	Negative
3	70°	„
6	70°	„
9	70°	Positive

No close reproducibility as regards the quantitative data could be obtained. Qualitatively the results are unequivocal. On aeration at 90° the addition of 0·001–0·02 % sodium stearate or 0·004–0·02 % sodium oleate regularly brings about a rapid formation of oxycholesterol. The residues obtained in these cases were quite transparent and only very faintly yellow. Higher soap concentrations were not tried on account of excessive foaming. If the temperature is lowered the rate of oxycholesterol formation is also lowered. There

seems to be a steep fall of the rate of oxycholesterol formation in the stearate experiments at 60–55° and in the oleate experiments at 80–70°, which is probably not compatible with a normal temperature coefficient of the rate of the reaction.

In a few experiments with sodium palmitate, potassium palmitate and potassium stearate no oxycholesterol was at first formed on aeration at 90°. As other experiments had shown that the addition of alkali inhibited the effect of sodium stearate on oxycholesterol formation, we added small amounts of sulphuric acid to the soap solutions used (the amount of acid being so small that the fatty acids were not precipitated and the solutions were still faintly red to phenolphthalein). After the addition of acid the soap solutions brought about a liberal formation of oxycholesterol, while the addition of alkali in excess also, in the case of these soaps, inhibited the oxycholesterol production.

IV.

The observation of the marked effect of the soaps led us to investigate the effect of adding other hydrophile colloids. Thus a series of proteins (glutin, edestin, ovomucoid, peptone) was tried, further two carbohydrates, dextrin and gum arabic and finally unsaturated phosphatides from egg-yolk. Of these substances the ovomucoid and the phosphatides were prepared by ourselves, the others were commercial specimens. The concentrations used were the same as in the soap experiments. The aerations were carried out at 90° and also at lower temperatures. In some experiments the hydrogen ion concentration was varied by addition of small amounts of acid or alkali. In no instance was any formation of oxycholesterol promoted. The addition of a protein to a cholesterol suspension was, indeed, found to inhibit the effect of soaps.

The easy oxidation of the cholesterol at temperatures above its melting-point led us to ascertain whether, in suspensions containing in the colloid state substances in which cholesterol is soluble, the latter was possibly prone to oxidation by oxygen. Accordingly mixed suspensions of different triglycerides and of higher fatty acids and cholesterol were prepared. In no instance was oxycholesterol formed on aeration at 90°. (As the fatty acids and the fats interfere with the Lifschütz reaction, the residues were in these cases dissolved in 5–10 cc. alcoholic sodium hydroxide, the alcohol was evaporated to dryness on the water-bath with exclusion of air and the residues were extracted with light petroleum to separate the soaps.) No oxycholesterol was formed on aeration of a mixed cholesteryl oleate-cholesterol suspension.

Considering the well-known rôle of many heavy metal salts as catalysts in oxidation reactions we tried the effect of the addition of small amounts of ferric chloride, ferrous sulphate and cupric chloride. As the cholesterol suspensions are very easily precipitated by addition of such salts, they must be used in very low concentrations. In a strength of 1/100,000 they had no effect on the formation of oxycholesterol in pure suspensions. On the addition of ferric chloride in 0·005 % and 0·001 % concentrations to cholesterol suspensions

containing 0·02–0·04 % phosphatides we observed a formation of oxycholesterol at room temperature and without aeration. The amounts of oxycholesterol formed were relatively small (5–25 %) and irregular, so that we do not regard these results as very definite. Aeration and higher temperature did not increase the oxycholesterol formation in these cases.

V.

The experimental results direct the attention to a certain physical factor which to all appearance is of the greatest importance for the attack of oxygen. It is very improbable that the soaps actually partake in the oxidation reaction. On the other hand the alkali soaps doubtless profoundly influence the colloidal state of the cholesterol. Simple inspection gives clear evidence of this fact. On adding (*e.g.*) 0·02 % sodium stearate to a 0·05 % cholesterol suspension, the mixture, when slowly heated, gradually becomes clearer. There is a distinct diminution of the turbidity at 40°, between 50–60° there is a further very marked clearing of the suspension, and at 60° it is nearly water-clear. Its turbidity equals then approximately that of a pure 0·05 % cholesterol suspension diluted with 7–8 parts of water. The clearness of the suspension contrasts strikingly with the milky appearance of a pure cholesterol suspension at the same temperature. When a cholesterol suspension containing 0·004 % sodium oleate is slowly heated, the turbidity remains practically unchanged up to between 70° and 80°, when it is rapidly reduced. (A 0·02 % sodium oleate solution itself is still rather milky at 90°.)

It should be noted that at the same temperatures at which the clearing of the suspensions takes place, there is—especially in the oleate experiments—a marked increase in the rate of the oxycholesterol formation, an increase which can hardly be an ordinary temperature effect on the rate of the reaction. It may also be mentioned that none of the other colloids tried brought about any visible clarification of the cholesterol suspensions.

There can be no doubt that this marked clearing of the suspensions is referable in the main to an increased degree of dispersion of the cholesterol. This is in itself no remarkable occurrence, the powerful peptising action of the alkali soaps being a well-known phenomenon. The point lies in the observation that parallel with the increased degree of dispersion brought about by the soaps the oxidisability of cholesterol by oxygen is essentially increased[1]. This observation suggests that, provided that the cholesterol exists in a sufficiently high degree of dispersion in water, the substance may require no particular catalyst for a relatively rapid oxidation by oxygen even at body temperature. As the cholesterol in the blood normally exists in a high degree of dispersion, the results obtained support to a limited degree the opinion that cholesterol

[1] In heterogeneous systems the rate of the reaction is a function of the surface area between the phases containing the reacting substances. The degree of dispersion, therefore, strongly influences the rate of the reaction. It can, however, not be excluded that the soaps act catalytically also in some other way than by changing the degree of dispersion of the cholesterol.

may undergo oxidative changes in the blood. The observed inhibition of the soap effect by alkali and the resistance of cholesterol in alcoholic solution against oxygen suggest, *inter alia*, that other factors than the degree of dispersion are of decisive importance.

With regard to the question of the catalysing substances present in impure cholesterol, there are certain evidences of their acting in the same manner as the soaps. The fact that with impure cholesterol it was possible to prepare more concentrated suspensions than with the quite pure substance points to the presence of peptising agents in the impure preparations. The non-formation of oxycholesterol on aeration at 37° for 48 hours (in the experiment of Moore and Willimott) as compared with the liberal amounts of oxycholesterol formed on direct evaporation of the suspensions on the boiling water-bath indicates an abnormal temperature coefficient of the rate of the reaction in the same way as in the soap experiments. Furthermore, in our first series of experiments (p. 1315) with impure cholesterol we often noted a very marked clearing of the suspensions in cases where oxycholesterol was formed. As soaps are claimed to be normal constituents of the bile there is the possibility that the active impurities (in our preparations) were actually soaps. The active principle of the brain extract was not investigated.

As to the failure to get quantitatively reproducible results we think that it is most likely due to the difficulty to reproduce exactly the same colloidal state (or series of states) in the different experiments.

The observations made call for caution against the action of oxygen in the determination of oxycholesterol in blood or tissues. Finally the relative character of the qualitative results obtained with the Lifschütz reaction may be emphasised. It is clear that if greater quantities of the suspensions had been used in the aeration experiments than those employed by us, positive Lifschütz reactions might have been encountered in cases where we obtained negative ones, and conversely if smaller quantities of suspensions had been taken negative reactions might have been found where we got positive ones.

Summary.

The formation of oxycholesterol on heating cholesterol in the air at temperatures slightly above the melting-point or on aeration of cholesterol suspensions is due to the action of the atmospheric oxygen. The observation by Robertson that suspensions of pure cholesterol are, even at boiling temperature, very stable against the oxygen of the air is confirmed. Impurities very stubbornly adhering to the cholesterol catalyse the formation of oxycholesterol on aeration. Alkali-soaps have a marked catalysing effect on the oxidation of the cholesterol by molecular oxygen. A series of other hydrophile colloids tried had no catalytic action. The same was the case with a series of higher fatty acids, triglycerides and heavy metal salts. The action of the soaps in all probability is mainly due to their peptising action on the cholesterol. The catalysing impurities probably act in the same way. The observations

made call attention to the great importance of the colloidal state of the cholesterol—especially its degree of dispersion—for the oxidisability of the substance by molecular oxygen.

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CLXVI. A NOTE ON QUANTITATIVE METHODS OF MEASURING THE NUTRITIVE VALUE OF PROTEINS.

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(*Received July 16th, 1928.*)

In an article appearing recently in this *Journal*, Kon [1928] has applied to the study of the nutritive value of the protein tuberin, the globulin of the potato tuber, the method elaborated by the author [1924], involving a study of the nitrogen metabolism of young growing rats under controlled conditions of feeding. In comparison with this method, analogous results obtained by a quantitative growth method devised by Osborne, Mendel and Ferry [1919] were also presented. According to the first method, the nutritive value of the protein is taken as the total nitrogen retention of the experimental animal in percentage of the total absorbed nitrogen. This percentage is called the "biological value" of the protein, a term introduced by Thomas [1909]. According to the second method, the nutritive value of the protein is measured by the gain in body-weight of the experimental animal per gram of protein consumed.

In discussing the significance of the results secured by these two methods, Kon makes certain statements to which exception may be taken. Speaking of the nitrogen balance method, he says (pp. 266–7): "A specific error seems to be inherent in the method... namely, the tendency to give higher biological values in the periods immediately following the standardising nitrogen-free or low-nitrogen periods. This is especially marked in the present work, but may also be found in the papers of Mitchell and his co-workers, especially Mitchell and Beadles [1927], and of Klein, Harrow, Pine and Funk [1926]."

If this were true it would constitute a serious criticism of the method. Among the first applications of the method in the author's laboratory, the possibility of such an error was anticipated and specifically studied by Mitchell and Carman [1924], with results quite contrary to the conclusion of Kon. It should be realised in a consideration of this question, first, that discrepancies will generally exist between duplicate determinations of the biological value of a specific source of protein obtained with two groups of rats or even with the same group of rats, and, second, that if one of the duplicate determinations is made in the period following a period of low-nitrogen feeding, the chances are even that it will be larger than the other determinations not so made,

even though the period of nitrogen underfeeding has exerted no effect on the utilisation of the dietary protein. In other words, the question is a statistical one, and cannot be settled by citing isolated instances in which experimental data *appear* to indicate an increased biological value following nitrogen underfeeding. The results of Kon obtained with tuberin are of this description, but neither they, nor the other results cited, are sufficient to prove the point at issue, since they have been selected from a number of other results of an opposite significance. Thus, in the work of Klein *et al.* [1926], wheat bran gave an average biological value of 60 following a period of nitrogen-free feeding, and one of 54 in another type of period, but corn meal gave very poor and variable results (averaging only 18) in a period following a standardising period, and an average of 56 in another period not so located. The same situation exists in the work of Mitchell and Beadles [1927] to which reference is also made.

It seems clear that the best solution of the question can be obtained only from a survey of all published data that might bear upon it. Besides the Illinois data, there are only those data from Cambridge and Warsaw already cited. The following table summarises all of the data obtained from the author's laboratory at the University of Illinois.

The effect of a period of low-nitrogen feeding on the biological value of protein obtained in an immediately following period.

Source of protein	Average biological values obtained: Following low-N feeding (1)	Average biological values obtained: Not following low-N feeding (2)	Excess of (1) over (2)	Reference
Whole egg	92	94	−2	Mitchell and Carman [1924]
Whole wheat	63	68	−5	,, ,, ,,
Cocoa + milk	73	65	+8	Mitchell, Beadles and Keith [1926]
Whole egg	90	99	−9	Mitchell and Carman [1926]
White flour	56	48	+8	,, ,, ,,
Egg albumin	86	81	+5	,, ,, ,,
Egg albumin	82	81	+1	,, ,, ,,
White flour	48	54	−6	,, ,, ,,
Veal	64	59	+5	,, ,, ,,
White flour	49	49	±0	,, ,, ,,
Beef + white flour	70	76	−6	,, ,, ,,
White flour	55	55	±0	,, ,, ,,
Beef liver	78	75	+3	Mitchell and Beadles [1927]
Beef heart	84	68	+16	,, ,, ,,
Beef heart	76	69	+7	,, ,, ,,
Beef liver	73	75	−2	,, ,, ,,
Pork tenderloin	79	74	+5	Mitchell, Beadles and Kruger [1927]

Among 17 possible comparisons, 9 favoured the view that low-nitrogen feeding increases the biological value in an immediately subsequent period, 6 contradicted this view, and 2 gave a non-committal answer. The average difference in biological value for the 17 cases was + 1·6, in favour of the value obtained after low-nitrogen feeding. As the method is applied in the author's laboratory, there seems to be no reason to believe that a period of low-nitrogen

feeding exerts any but an inappreciable effect upon the utilisation of protein in a subsequent period.

Kon is also in error in his attempt to explain differences in the nutritive ratings of proteins as obtained by the author's method and by the method of Osborne, Mendel and Ferry. On this point he says: "Obviously the two methods are not strictly comparable, as the one measures the efficiency of a given protein to make good, over short periods of time, the wear and tear of body tissues, while the other measures the ability of a protein to build new tissues."

This constitutes a misinterpretation of the significance of results secured by the author's method, a misinterpretation that can be readily refuted by any of the calculations made by Kon himself. Take the data of Rat 1 on the tuberin ration, for instance. This rat consumed daily 104 mg. of tuberin-nitrogen and excreted daily in the faeces 23 mg. of nitrogen, of which 22 mg. were estimated to be of body origin (metabolic nitrogen). Hence, only 1 mg. of dietary nitrogen was actually indigestible, and the absorbed nitrogen, therefore, amounted to 103 mg. daily. The daily urinary nitrogen amounted to 45 mg., of which 20 mg. was estimated to be of body origin (endogenous) and only 25 mg. represented unutilised dietary nitrogen. Hence, of the 103 mg. of tuberin nitrogen absorbed 25 mg. was wasted and 78 mg., or 76 % (the biological value), was retained in the body. According to Kon, this 78 mg. was used only to make good the wear and tear of body tissues. But this rat was growing and storing daily 36 mg. of nitrogen ($104 - 23 - 45 = 36$), which must be an integral fraction of the total retention of 78 mg., since no other source of nitrogen was available. The latter value includes all functions, maintenance, growth, and the replenishment of the digestive glands—for which the dietary nitrogen is being used.

Thus, the biological value of a protein measures that fraction of the absorbed protein nitrogen that is being used in all of the anabolic reactions of the body. On the other hand, the gain per gram of protein consumed obtained under the conditions prescribed by Osborne, Mendel and Ferry credits to the dietary protein only that fraction of the protein intake that is being used for growth. Hence, this measure of the nutritive value of a protein is affected, not only by the amino-acid make-up of the protein itself and the unknown physiological factors that determine its utilisation in anabolism, but also by the ratio of protein consumed to the size of rat used. The larger this ratio becomes, the smaller will be the fraction of the nitrogen intake needed to make good the endogenous losses of nitrogen, and the greater the fraction that is available for growth, and, hence, for the same source of protein, the greater the gain per gram of protein consumed. For this reason, this measure of protein value is subject to variation caused by variable food intakes. If rats of equal size are employed and the food intakes are comparable, the measures of protein efficiency obtained for different proteins will be comparable. But it is a hazardous undertaking to compare gains per gram of protein consumed

obtained in different experiments without reference to the size of animals employed, or to the amounts of food consumed, as Kon and others before him have done.

Hoagland and Snider [1926], in one of their first applications of the method of Osborne, Mendel and Ferry to the study of the nutritive value of proteins, noted that, throughout a number of ration comparisons, the gain in weight per gram of protein consumed was in the large majority of cases greater for the male than for the female rats. This sex difference was correctly interpreted as the result of the greater growth capacity of male rats and the resulting greater consumption of food in proportion to weight. Hence, with the males a larger proportion of the protein consumed was available for growth and was so used. Although in their later work Hoagland and Snider used only male rats, the complete significance of these early experiments has not been appreciated, *i.e.* that in comparisons of different sources of protein a great disparity in the food consumption of experimental rats, whether because of differences in sex or of the growth-promoting value of the rations, will always obscure the interpretation of the observed differences in the ratio of gain to protein consumed.

There is another reason why the results obtained by the author's method for a series of proteins will differ from those obtained by the numerical method of Osborne, Mendel and Ferry. In the former method differences in the digestibility of proteins are not involved in the biological values obtained, while in the results of the latter method they are. For two proteins of equal biological value, the gain in weight per gram consumed will be the greater for the more digestible protein. This is one reason why animal proteins are graded so much higher than cereal proteins by this method than they are by their respective biological values.

Either method is capable of yielding accurate comparisons of the nutritive values of different sources of protein, but only when the necessary experimental conditions are imposed, as dictated by a critical study of all factors that may affect the final results. However, the methods are not equivalent and the results obtained need not parallel each other.

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CLXVII. OXIDISING ENZYMES.

X. THE RELATIONSHIP OF OXYGENASE TO TYROSINASE.

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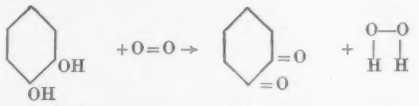
(*Received September 3rd, 1928.*)

In a recent communication [Pugh and Raper, 1927], the following statement has been made: "tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative....There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow." The present communication records some observations made in connection with the above statement.

It is known that a preparation from the potato and many other Higher Plants giving the "direct oxidase" reaction made according to Onslow [1920], that is, by thorough extraction of the tissues with cold alcohol without undue oxidation, and subsequent extraction of the enzymes with water from the residue, will rapidly convert catechol and other *o*-dihydroxy-substances into *o*-quinones [Szent-Györgyi, 1925; Pugh and Raper,[1] 1927] and the monophenols, phenol, *p*-cresol, *m*-cresol and tyrosol, and *p*-hydroxybenzoic acid [Onslow and Robinson, 1925; Uys-Smith, 1926; Pugh and Raper,[1] 1927] and tyrosine [Onslow, 1923; Raper,[2] 1926] into the corresponding *o*-dihydroxy-compounds, with the subsequent formation of *o*-quinones. Such a preparation also contains peroxidase.

It is known [Onslow, 1920] that if an aqueous solution of catechol is exposed to air, it autoxidises with the formation of a peroxide, since it reacts with peroxidase and guaiacum. As far as qualitative tests are valid, the peroxide appears to be hydrogen peroxide on account of the reaction given with titanium sulphate [Onslow and Robinson, 1926; Platt and Wormall, 1927]. It is possible that *o*-quinone and hydrogen peroxide are formed according to the equation below:



[1] The *o*-quinone was identified by Raper using tyrosinase from the meal-worm, and catechol, phenol, *p*-cresol and *m*-cresol as substrates.

[2] 3 : 4-Dihydroxyphenylalanine was also identified by Raper in this reaction, again using tyrosinase from the meal-worm.

The *o*-quinone here, however, cannot be detected by guaiacum. It has been suggested [Szent-Györgyi, 1925] that it may condense under certain conditions with unchanged catechol.

Such a reaction now appears to represent that carried out by the enzyme, oxygenase, of the Higher Plants, the *o*-quinone having recently been isolated by Pugh and Raper (as stated above), who have, moreover, shown that the same product is formed from catechol by the action of peroxidase (horse-radish) and hydrogen peroxide.

Since such oxygenase plants as have been examined also contain a catechol derivative [Onslow, 1921], it is reasonable to assume that the above is the reaction which takes place, with simultaneous browning, on injury to the cell.

Now, the preparation as described by Onslow contains oxygenase and peroxidase, both of which can carry out the reaction denoted above. It is obvious, moreover, that such a preparation may always contain traces of *o*-quinone, though not sufficient to blue guaiacum, and possibly of catechol. The oxidation of catechol in the presence of such a preparation is therefore due to both oxygenase and to peroxidase and hydrogen peroxide; in addition, the oxygenase itself might be regarded essentially as the *o*-quinone, perhaps stabilised by association with a colloidal surface. The question also naturally arises as to whether the oxidation of the monophenols is brought about by the same enzyme as that which catalyses the oxidation of *o*-dihydroxy-compounds or by another catalyst; or whether it is a secondary oxidation in which *o*-quinone, formed from the diphenols originally present, takes part. Bearing in mind, therefore, that the *o*-quinone may be an intrinsic part of the oxidising system, we have tried experimentally to obtain some evidence on this point.

On this assumption, a plant tissue which has been allowed to oxidise should yield a more active enzyme preparation than one which has not received such treatment, because, in the former, more *o*-quinone will presumably be present. We therefore took two small portions (about 20 g. each) from the same potato. One portion was coarsely pounded in a mortar and allowed to oxidise for about 10 minutes; it was then thoroughly ground and extracted five times with alcohol and air-dried. The other portion was very rapidly sliced under alcohol with maximum care to avoid contact with air, and immediately ground and extracted with alcohol five times and air-dried. Equal portions of both preparations were then treated with equal volumes of freshly-prepared 0·1 % aqueous catechol, 0·1 % *p*-cresol in phosphate buffer (p_H 7·3), and a saturated solution of tyrosine in the same buffer. The oxidised preparation acted more rapidly on all three substances, the differences in rate being small but definite for catechol, and greater for *p*-cresol and tyrosine. We have so far been unable to maintain this difference on reprecipitation of the two enzyme preparations from aqueous solution by alcohol.

In addition, we have tried the effect of treatment of the enzyme preparation with charcoal, with the object of removing the traces of *o*-quinone present. About 75 g. of potatoes were pounded, extracted four times with alcohol and

air-dried. The residue was then extracted with 50 cc. of water, and filtered, about 40 cc. of extract being obtained. Preliminary tests with freshly prepared aqueous catechol, and with *p*-cresol and tyrosine, both in buffer solution, were made; the solution was then shaken up five times with Merck's charcoal (0·1 g. per 5 cc.), portions being tested between each adsorption. The results obtained are set forth in the following table.

Solutions tested with 0·5 cc. enzyme extract		Original extract	Extract after 1st adsorption with charcoal	After 2nd adsorption	After 3rd adsorption	After 4th adsorption	After 5th adsorption
0·1 % catechol	1 cc.	Instantaneous	Instantaneous	Instantaneous	Instantaneous	1 min.	3 min.
0·1 % *p*-cresol Water	1 cc. 1 cc.	3 min.	42 min.	55 min.	85 min.	Negative after 2 days	Negative after 2 days
0·1 % *p*-cresol 0·0005 % catechol	1 cc. 1 cc.	—	4 min.	5 min.	8 min.	23 min.	>1 hour colour fainter
Sat. solution tyrosine Water	1 cc. 1 cc.	50 min.	Negative after 2 days	Negative	Negative	Negative	Negative
Sat. solution tyrosine 0·0005 % catechol	1 cc. 1 cc.	35 min.	6 min.	10 min.	15 min.	>1 hour	Negative

The times recorded refer in each case to the first appearance of a definite colour.

It will be seen from the table that the onset of oxidation of catechol was not obviously retarded until after the fourth adsorption, although the intensity of the reaction, as indicated by the colour, gradually decreased. The oxidation of *p*-cresol, on the other hand, was diminished very considerably after the first adsorption and eventually ceased, whereas it was restored to practically the original value by the addition of about 0·005 mg. of catechol. The extract was unable to bring about the oxidation of tyrosine after the first adsorption; this capacity was restored to a small extent by the addition of traces of catechol; unlike, however, the case of *p*-cresol, oxidation does not proceed normally, a very small amount of the tyrosine present being oxidised.

From the observations recorded above, we conclude that the activity of the colloidal constituent is at first almost unimpaired by charcoal, as shown by the continued rapid reaction with catechol. On the other hand, the oxidation of *p*-cresol is greatly retarded by the first adsorption with charcoal, and gradually ceases with successive treatments; it is, moreover, restored to almost its initial strength by the addition of a trace of catechol. It may be assumed, therefore, that it is a secondary oxidation depending on the presence of *o*-quinone, which has been largely removed by the first, and probably completely so, by the fifth treatment. It seems as if the addition of a trace of catechol may result in the production of *o*-quinone, which initiates the autocatalysis following on the induced oxidation with production of homocatechol from *p*-cresol.

In the case of tyrosine, induced oxidation seems to take place to a certain

extent, after adsorption, on the addition of traces of catechol, as the solutions turn pale pink and darken slightly; but, as the reaction never increases in intensity, autocatalysis apparently does not proceed far under these conditions. It is known from the work of Raper [1927], that further oxidations take place in the formation of melanin from tyrosine, and the reaction is obviously more complicated than in the case of *p*-cresol.

Peroxidase has always been detected in our enzyme extracts after the last adsorption. The presence of peroxidase has a certain significance in the experiment, for, as stated earlier in the paper, a solution of catechol slowly autoxidises in air, producing, in all probability, hydrogen peroxide. In the presence of the latter and peroxidase, *o*-quinone is formed from catechol. The difference in effect between a freshly prepared catechol solution and one which has stood for 24 hours or more could be demonstrated, not only with the original enzyme extract, but also after each successive adsorption, the colour appearing slightly earlier with the autoxidised catechol.

It appears to us to be certain that there is an enzyme, present in plants giving the "direct oxidase reaction," which catalyses the autoxidation of *o*-dihydroxy-compounds with the probable production of hydrogen peroxide and *o*-quinone; the plants which give the "peroxidase reaction" only are incapable of originating such a catalysis. We see no reason for discarding the term oxygenase for this enzyme, or for substituting the term tyrosinase for an enzyme which primarily catalyses the oxidation of substances with the *o*-dihydroxy-grouping. From the evidence given in this paper, it seems highly probable that the *o*-quinones produced from such substances will bring about, as a secondary oxidation, the formation of dihydroxy-compounds from monophenols, such as *p*-cresol. To a slight extent the *o*-quinone can initiate the formation of melanin from tyrosine, but the fact that the oxidation is not greater in extent may indicate that some additional factor is concerned in the normal reaction. It seems, nevertheless, that the term, tyrosinase, may, when the conditions are definitely realised, ultimately come to be interpreted as oxygenase plus an *o*-dihydroxy-compound or its derived quinone. If, in addition, it should be proved that the oxygenase itself is really an *o*-quinone in conjunction with a colloidal surface, then the original conception of Bach and Chodat [Chodat, 1910] of the oxygenase as "an enzyme-like peroxide" would be vindicated, though re-expressed in more modern terminology. Throughout the present paper, our remarks only apply to enzymes of the Higher Plants. Further, we have been unable to demonstrate a tyrosinase reaction in any plant which did not also contain the catechol-oxygenase system. In regard to the polyphenolase and laccase oxidising systems of the Higher Plants, our view is that these terms are synonymous with the oxygenase-catechol system. It is true that certain laccases of the Higher Plants [Bertrand, 1896] have been stated to have no action on tyrosine. We also have found a few cases of oxygenase-catechol plants not acting on tyrosine; but, as the conditions of the reaction are not completely understood, they cannot yet be

established as definite exceptions. We have retained the name, oxygenase, on account of the classical terminology of Bach and Chodat. Until it should be known definitely that oxygen is not activated in the reaction, we see no reason for substituting another term.

Concerning the classification of aerobic oxidases proposed by Pugh and Raper [1927], we would like to point out, in connection with system (1), namely peroxidase plus an autoxidisable substance [Gallagher, 1923], that a criticism of the interpretation of this author's observations has been published by one of us [Robinson, 1924]. The work of Euler and Bolin [1908], which concerns the proposed system (3), has been criticised by Bunzel [1915]. System (4), tyrosinase, we prefer to interpret on the lines indicated above, where we have expressed our views as to the connection of this enzyme with the catechol-oxygenase, the polyphenolase and the laccase systems.

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CLXVIII. GLUCOSE IN NORMAL URINE.

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(*Received July 13th, 1928.*)

Introduction.

In spite of considerable investigation, the question whether small amounts of glucose may occur in normal urine has not yet been satisfactorily answered. The present state of indecision is partly due to the absence of a specific test for this sugar and partly to the fact that it normally occurs in very small quantities.

Apart from reduction tests the detection of glucose has generally been based on (1) fermentation with yeast, and (2) the phenylhydrazine reaction. That the reaction is not specific for glucose is not the only drawback as far as yeast fermentation is concerned, for yeast [von Lipmann, 1904] fails, sometimes, to ferment sugar when the concentration of the latter is as low as 0·1 g. per 100 cc. During the present investigation this observation was confirmed. It was possible to obtain glucosazone from urines to which 0·1 g. glucose per 100 cc. had been added even after 3 days' incubation at 37° with a pure yeast culture [see also Patterson, 1926].

Objections have often been raised against the use of yeast for the detection of glucose in normal urine. The laborious technique, which the use of pure cultures of yeast necessitates, has unfortunately led to the employment of ordinary yeast instead. This practice has no doubt weakened the evidence for the existence of glucose, though in the majority of urines thus treated fermentation did occur [Benedict and his co-workers, 1918; Sumner, 1921; Neuwirth, 1922]. But, as has frequently been suggested, such fermentation might be due to the bacteria introduced with the yeast. To attempt to inhibit bacterial growth by making the urine slightly acid or by adding a little volatile antiseptic like toluene is of doubtful value especially if, as is customary, fermentation with ordinary yeast is allowed to go on for 17 to 24 hours or more. In spite of starting with a p_H varying between 5·5 and 6·8, Höst [1923] found that some of the urines undergoing fermentation with ordinary yeast became strongly ammoniacal. Much reliance cannot, therefore, be placed on positive evidence obtained in experiments with yeast if bacteria were present.

The osazone reaction properly carried out is free from such serious drawbacks, and it may, therefore, be expected to furnish more reliable information regarding the question of glucose in normal urine. As has been pointed out

by Höst [1923], the only sugar capable of giving an osazone identical with that of glucose is laevulose, but the optical behaviour of normal urine seems to show that laevulose is most probably not one of the urinary sugars. Moreover, observations reported by Folin and Berglund [1922] suggest that, unlike galactose and lactose, laevulose ingested even in large quantities is retained in the body.

The belief that glucose is a constituent of normal urine arose from observations made by Baisch [1894] and confirmed by Lemaire [1895] and by Pavy and Siau [1901]. These investigators isolated from normal urines an osazone apparently identical with glucosazone. Recently, however, Geelmuyden [1915] and Höst [1923], who applied the same test to small quantities of urines, were unable to confirm the results arrived at by these earlier investigators, though many normal urines were examined.

Dissatisfied with the methods so far employed, Folin and Berglund [1922] attempted to throw fresh light on the problem by studying the relation between sugar elimination and the concentration of sugar in the blood. The blood-sugar and the urine-sugar were determined before and after hydrolysis under various conditions and it was found that giving as much as 200 g. of pure glucose to normal persons was not followed by any glucosuria, whereas definite "glycuresis" was obtained after every ordinary carbohydrate meal. These observers ascribe the increase in the reducing substances of the urine after an ordinary meal to the excretion of foreign unassimilable carbohydrates and carbohydrate decomposition products produced during the preparation of the food. According to them glucose is probably not one of the urinary sugars. Similar conclusions were arrived at by Höst [1923] and by Greenwald and his co-workers [1925].

The present paper records certain observations which were made while applying the osazone test to a number of urines mainly from healthy Egyptian students and which provide confirmation of the older view that glucose may be a constituent of normal urine.

Experimental.

1. *The preparation of the osazones of the urinary sugars.*

Normal urines contain substances that interfere with the proper crystallisation and consequently with the identification of the osazones of the urinary sugars. Even when the concentration of the sugars is so large that recognisable crystalline osazones come down when the urine is treated with phenylhydrazine, the crystals are, as a rule, ill-formed, dirty and often buried in an amorphous deposit of brown particles and oily drops. The substances which thus interfere with the formation and crystallisation of osazones can be removed quite readily by treating the urine with adsorbent charcoal before applying the phenylhydrazine test.

In all the experiments recorded in this paper, the urines were treated in this manner and evidence was obtained that charcoal removes these interfering substances without appreciably lowering the concentration of the urinary sugars. The charcoal employed was usually unpurified Merck's decolorising charcoal. The following method was found suitable for the preparation of osazones from normal urines.

25 cc. urine were well mixed with 2 g. dry charcoal and filtered after standing for about 5 minutes. About 15–17 cc. clear colourless filtrate was usually obtained, and to this was added 1 g. of a mixture of pure phenylhydrazine hydrochloride and sodium acetate (prepared by mixing well in a mortar one part of pure phenylhydrazine hydrochloride and two parts of sodium acetate just before use). The filtrate was now placed in a boiling water-bath for about 5 minutes or just until the reagents were dissolved and then filtered into another clean tube. The final filtrate was put back in the bath for 2 hours and then allowed to cool gradually in the bath and the deposit examined for crystals 12–15 hours later.

As described above the reaction is not only sensitive but seldom fails to yield clean and well-crystallised osazones. Occasionally urines of low specific gravity fail to give crystals at first, but most of these do so on longer standing at a temperature of 3–5°.

Sometimes the crystals are contaminated with variable amounts of orange-brown oily drops which, whilst in no way interfering with the delicacy of the test, may tend to obscure their appearance under the microscope. This impurity can be effectively removed by washing the deposit two to three times with a little ether after the removal of the supernatant liquid.

The sensitivity of the osazone test.

To test the delicacy of this reaction for the detection of glucose, it was applied, in the form described above, to solutions of glucose in water and in urine.

Glucose dissolved in water. From aqueous solutions of glucose it was a simple matter to obtain typical glucosazone crystals when the concentration of glucose was 2·5 mg. per 100 cc. This confirms the observations of Höst [1923] who employed a modified Neumann's method. The reaction is, however, more sensitive than he imagined, for by cooling the tubes to 3–5° for a few hours, aqueous solutions containing as little as 1 mg. glucose per 100 cc. were found to give crystals of glucosazone.

Glucose added to urine. The presence in urine of substances which interfere with the osazone test, as well as the fact that all urines examined yield osazones, make it difficult to determine the delicacy of the test for the detection of added glucose until the urinary sugars have been removed. This was accomplished by bacterial fermentation in the following way.

The urine is made just alkaline with ammonia, inoculated with a few drops from another urine undergoing active alkaline fermentation and incubated at

37° for 10–12 hours. It is then acidified slightly with acetic acid, a sufficient quantity of washed kieselguhr added to assist the flocculation of the bacteria and brought up to the boil. After cooling, it is filtered and the clear filtrate is used for the test. Such urine will not give an osazone.

Tests on such urines with added glucose demonstrated that as little as 1 mg. per 100 cc. can be detected by the osazone test.

The nature of the osazones derived from normal urine.

The crystalline forms of the osazones obtained from normal urines are very varied (Plate V, Figs. 1–7). Nevertheless, it can be shown that they usually consist of mixtures of at least two osazones which can, in most cases, be separated by simple means. The crystals may be composed entirely either of fine needles or of broad flat needles, but more frequently both kinds are combined to form one crystal mixture (Figs. 2, 3 and 4). In some, however, their mixed nature is not quite so clear. Union between the two osazones apparently takes place before crystallisation and the resulting crystal possesses new characters, but these are always intermediate between those of the components (Fig. 5). A more complex type of crystal is frequently met (Fig. 6) but this is simply an aggregate of crystals belonging to the two classes of the crystal mixture just described.

The appearance of crystals in Figs. 2, 3, 4 and 6 indicates that they are a mixture of the two osazones represented by Figs. 1 and 7. This can be proved by fractional crystallisation, since these two osazones differ markedly in solubility.

The osazone deposit from 200 cc. of a 24 hours' fresh urine was washed with distilled water and then with ether, 10 cc. of 20 % alcohol were then added and after stirring well, the mixture was centrifuged in a corked tube. The clear orange-brown supernatant liquid was then drained off and kept aside and the residue examined microscopically. This residue was dissolved in a little hot absolute alcohol and distilled water carefully added to the solution until a slight permanent turbidity occurred. The solution was then boiled to drive off some of the alcohol and on cooling fine long needles were obtained, many of which remained single, others formed broom-like aggregates. Microscopically these crystals were identical with glucosazone.

The clear supernatant liquid was treated with a little ether, and the top ether-alcohol layer pipetted off. This on recrystallisation gave a mixture of crystalline forms in which were recognised (*a*) a small glucosazone sheaf, (*b*) a larger irregular mixed osazone sheaf, and (*c*) two separate broad flat needles.

Crystals containing comparatively little glucosazone give after such treatment a fair yield of the osazone which crystallises out in broad flat needles.

The fractional crystallisation of many similar crystals obtained from urine gave only the following two simple osazones.

Osazone I. This is characterised by crystallising in broad flat orange-yellow needles of which the ends usually taper gradually, or in some cases form blunt spear-shaped points. The needles tend to form aggregates usually in the form of large irregular rosettes. Microscopically the crystals sometimes resemble maltosazone, but their melting-point is much lower, 152–153°. This osazone is soluble in hot water and in 20 % alcohol (Fig. 1).

Osazone II. Glucosazone. This is characterised by its fine long dark yellow needles which occur singly or more often in sheaves, brooms and rosettes. When formed from urine this osazone tends to float to the surface of the liquid forming a woolly mass in which crystals belonging to other types may be found entangled. If the amount is small, this osazone remains at the bottom among the deposit formed by the other crystals. When purified by solution in hot absolute alcohol and recrystallisation from dilute alcohol (about 20 %) this osazone melts at 202–204°, but when first obtained from urine its melting-point is much lower, 188–198°. The purified osazone is insoluble in hot water and in cold absolute alcohol but dissolves in the latter on heating. It appears to be identical with glucosazone in melting-point, colour, crystalline appearance and solubility. Fig. 7 represents a sheaf obtained from a normal urine voided 1½ hours after an ordinary meal.

The crystal mixtures have no fixed melting-point, solubility or crystalline form. These characteristics depend entirely on the amount contributed to the crystal by each osazone. This peculiarity might explain why so many investigators succeeded in obtaining from normal urines osazones with various melting-points and crystalline forms. But it is quite significant that the melting-point of all these new osazones lies between those of the two osazones already described. It is, therefore, highly probable that all such osazones are of the same nature as the crystal mixtures. The osazone isolated by Patterson [1926] seems to be a good example, for its crystalline form resembles closely certain crystal mixtures which can be easily prepared by dissolving a mixture of osazone I and osazone II in a little hot absolute alcohol and then fractionating it by simply diluting with water. As the alcohol becomes weaker, two to three well-formed precipitates come down. On examination the appearance and properties of these fractions will be found to be intermediate between those of the components.

The principal object of this investigation being to throw some light on the long-discussed question of the occurrence of glucose in normal urines, little attention has been given to the identity of osazone I. Baisch [1895], however, isolated from urines fermented with yeast an osazone which had M.P. 152–154°, and Lemaire [1895] later obtained a similar product of M.P. 150–151°. The former investigator believed his osazone to be identical with Fischer's *iso*-maltosazone both in crystalline appearance and melting-point, but apparently there has been no further evidence provided regarding the identity of Baisch's product. It is uncertain whether the product is really *iso*-maltosazone or not. The melting-point of the product isolated by Baisch and Lemaire agrees with

that given for osazone I, but the crystalline appearance of osazone I is totally different. For, while the osazone isolated by these observers crystallises in small rosettes composed of very fine needles, much finer than those of glucosazone, the needles of osazone I are broad, flat and large. This, however, may be due to differences in the method of preparation. An osazone prepared from a specimen of *iso*-maltose kindly supplied by Prof. Ling gave crystals similar to those described by Baisch and Lemaire, and when glucosazone was added to it and then recrystallised, crystal mixtures were obtained, but none of the crystals resembled osazone I.

Crystals similar to those described here for osazone I have also been obtained by Geelmuyden [1915] from normal urines and from urines of "sugar-free" diabetics.

The detection of glucose added to ordinary urine.

If glucose is added to ordinary urines the quantity that gives typical glucosazone will, as Höst [1923] pointed out, vary. Höst obtained typical glucosazones from ordinary urines after adding about 10 mg. glucose per 100 cc. and in some cases not before the addition of 20 mg. The causes of these variations may be appreciated from what has already been stated regarding interfering substances. There is little doubt that the tendency of the osazones of the urinary sugars to form crystal mixtures is responsible for these variations. Variable crystalline forms with mixed types of crystals are also obtained when aqueous solutions of mixed sugars are examined. From an aqueous solution containing glucose and lactose it sometimes happens that nothing but typical glucosazone is obtained. But this glucosazone though typical microscopically is a crystal mixture from which the lactosazone can be recovered by fractional crystallisation. This behaviour explains why the majority of diabetic urines give only glucosazone crystals, and also explains the disappearance of the "physiological osazone" when the amount of glucose added by Höst to ordinary urines was adequate to produce "typical" glucosazone. The melting-point of such a typical glucosazone obtained from urine is generally much lower than that of pure glucosazone, because of the other osazone incorporated in it. The elimination of the latter osazone, by fractional crystallisation, leads to a marked rise in the melting-point. In a particular case the melting-point went up from 188° to 202°, or just two degrees less than that of pure glucosazone. It is not always easy to separate osazone I in a pure state from this typical glucosazone, but this point may be said to have been satisfactorily proved, if the rise in the melting-point follows the elimination of an osazone whose characters are unmistakably different from those of pure glucosazone.

It appears, therefore, that the phenylhydrazine reaction is less sensitive for the detection of glucose added to ordinary urines. This is, however, not entirely correct for small quantities of added glucose may be detected by watching the minute changes in the crystal mixtures, as little as 2·5 mg.

glucose per 100 cc. producing a visible increase in the glucosazone element in some of the crystal mixtures.

The phenylhydrazine reaction employed by Höst for the detection of glucose in normal urine.

Höst [1923] employed a modified Neumann's phenylhydrazine reaction and no precautions were taken to remove interfering substances from the urine. The osazones obtained by Höst from normal urines differ enormously in appearance from those described here. When his method was applied to 14 urines, no glucosazone whatever was obtained, though two of these urines gave typical glucosazone by the method described here. Some of the "physiological osazones" described by Höst were subjected to fractional crystallisation and in many cases it was possible to split them into glucosazone and another osazone very similar to osazone I. It, therefore, seems that Höst's failure to obtain glucosazone from normal urine was not due to the absence of glucose but to the method employed.

The occurrence of glucose in the urines of healthy individuals.

The osazone test in the form described here was applied frequently to the urines of about 700 Egyptians. These were mostly students whose ages varied between 17 and 27 years. Every urine gave osazone crystals that differed in detail in different persons and even in the same person at different times. Great variety of crystal shapes was encountered but nevertheless they were all mixtures of the types already described. The frequency with which typical glucosazone crystals were obtained was rather striking. Of urines voided 4–5 hours after a mixed meal 12–15 % give typical glucosazone crystals as well as other crystals. The remaining urines give crystal mixtures in which the glucosazone element varies greatly. In urines voided 1–2 hours after an ordinary meal the percentage is practically doubled (20–30 %) and the crystal mixtures in all the urines show a definite increase in the glucosazone element over what is usually obtained 4–5 hours after a meal. After fasting for about 12 hours the urines of two out of 30 persons gave typical glucosazones.

The question now arises as to the state of the carbohydrate metabolism in those whose urine gives typical glucosazone. Two points should be grasped before this question is discussed. Urines giving typical glucosazone do not reduce Fehling's reagent or Benedict's reagent in the ordinary sense. Such urines may give a reaction similar to that given by normal concentrated urines if equal parts of the reagent and the urine are boiled for 20 seconds and allowed to stand for 10–30 minutes. Again every subject examined passed at times a urine that gave typical glucosazone. The relation of this to meals was not always clear for it happened even after a 12 hours' fast.

To clear up this point, the blood-sugar and the osazones of the urine-sugars were simultaneously studied in 28 student volunteers, just before and after giving each 50 g. pure glucose dissolved in 100 cc. water by the mouth after

fasting for about 12 hours. Samples of urine and blood were taken just before administering the glucose and at half hourly intervals afterwards. The fasting urine of two students gave typical glucosazone, all the others gave crystal mixtures. After the glucose none of the urines gave typical glucosazone even in the case of the two students whose fasting urine gave typical glucosazone. The behaviour of the blood-sugar was apparently quite normal, for the fasting levels varied from 0·078 to 0·120 g. % and the maximum from 0·120 to 0·198 g. % and the return to the fasting level occurred from $1\frac{1}{2}$ to $2\frac{1}{2}$ hours after taking the glucose.

It is justifiable to believe, then, that when urines give typical glucosazone the condition is not always due to an abnormal carbohydrate metabolism. This statement applies to specimens of urine taken at random during the day, for it has always been observed that a mixed 24 hours' urine of a healthy individual will only give crystal mixtures but no typical glucosazone. Under normal conditions the balance between the urine-sugars in a mixed 24 hours' urine appears to be such that glucose does not have a chance of forming typical glucosazone.

It is interesting to note that the excretion of glucose after 50 g. pure glucose seems to be less than that which follows an ordinary mixed meal.

Discussion.

The failure of certain investigators, in particular Geelmuyden [1915] and Höst [1923], to obtain glucosazone from normal urines is probably due to the fact that the test is not sufficiently delicate to detect minute amounts of glucose unless certain precautions are observed. The most important of these is the removal by adsorption on charcoal of certain substances which interfere with the formation of the osazones. The tendency of different osazones to form crystal mixtures is another disturbing factor, which must be borne in mind when glucosazone is looked for. With the exception of crystals (*a*) and (*b*) in Geelmuyden's figure, and the few detached broad needles lower down in the diagram and also crystal (*m*), Geelmuyden's other crystals appear to be all of the mixed variety.

The results presented in this paper leave little doubt that glucose is a constituent of normal urine. Evidence for this can also be obtained from Höst's own results, for he observed that urines fermented with yeast required the addition of more glucose in order to give typical glucosazone crystals than unfermented urines. Höst himself suspected that this result might have the interpretation given to it here.

The increase in the reducing power of urines after ordinary meals seems to be due, at least in part, to an increased excretion of glucose and not, as has been suggested by Folin and Berglund [1922], to the presence of unassimilable carbohydrates and degradation products arising from carbohydrates during the preparation of the food. The paradoxical behaviour of the kidney towards glucose taken alone and that absorbed during the digestion of a mixed meal,

may have a great deal to do with the other digestion products absorbed with the glucose. Hamburger observed that changing the inorganic composition of a perfusate altered the permeability of the frog's kidney towards glucose.

Summary.

1. A trustworthy method for the preparation of the osazones of the urine-sugars is described.

2. When this method is applied to aqueous solutions of glucose or to glucose dissolved in urines from which the normal sugars have been removed, glucosazone is obtained even when the concentration is as low as 1 mg. per 100 cc. In suitable cases glucose added to ordinary urines can be detected when the amount added is as low as 2·5 mg. per 100 cc.

3. Normal urines give numerous types of crystal mixtures, but these have been shown to be formed of a mixture of two simple osazones, one of which is identical with glucosazone and the other appears to correspond with Baisch's *iso*-maltosazone, as regards melting-point, but differs markedly in crystalline appearance.

4. The urines of over 700 persons have been examined under different conditions, and it has been found that typical glucosazone crystals are given by 20–30 % of the urines voided 1–2 hours after an ordinary meal; the percentage drops to 12–15 in urines passed 4–5 hours after meals, and it falls further to approximately 7 % after a 12 hours' fast. That this is, in most cases, not due to an abnormal carbohydrate metabolism, has been shown by examining the tolerance of 28 students to 50 g. glucose.

5. Evidence is given to show that some of Höst's "physiological osazones" are impure crystal mixtures. It is also suggested that the same applies to some of Geelmuyden's osazones.

6. The old view that glucose in small quantities is a constituent of normal urine is confirmed.

I wish to thank Prof. J. C. Drummond for his kindness and advice. My thanks are also due to Dr F. N. Yassa and A. A. Hassan for helping me with the sugar tolerance tests.

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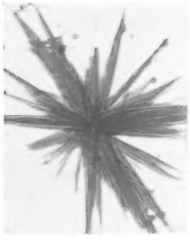

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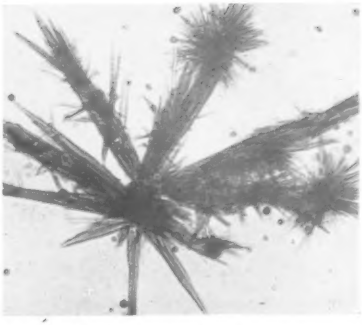

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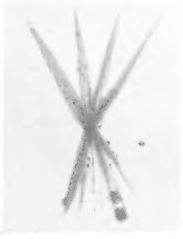

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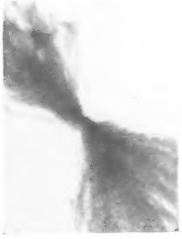

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Fig. 1. Osazone I. M.P. 152–153°.
Figs. 2–6. Crystal mixtures.
Fig. 7. Glucosazone "impure."

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